Chromatin Reprogramming During the Somatic-to-Reproductive Cell Fate Transition in Plants

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Zusammenfassung

Der Lebenszyklus von Blütenpflanzen ist gekennzeichnet durch den Wechsel zwischen einer diploiden sporophytischen und einer haploiden gametophytischen Generation. Die Entwicklung der gametophytischen Generation beginnt erst mit der Spezifizierung von Sporenmutterzellen (SMZ) während der Blütenentwicklung. Im Unterschied dazu wird die Keimlinie in Tieren bereits während der frühen Embryonalentwicklung angelegt. Die weibliche SMZ, oder Megasporenmutterzelle (MMZ), stammt von einer supepidermalen Zelle der weibliche Samenanlage (Ovule) ab. Demgegenüber differenziert sich die männliche SMZ, die Pollenmutterzelle (PMZ), in den sporogenen Geweben der Antheren. Die Spezifizierung der MMZ sowie der SMZ kennzeichnen den Übergang von somatischem zu reproduktivem zellulärem Schicksal. Um die haploiden Gametophyten zu bilden in denen sich die Gameten differenzieren, teilen sich die SMZ zunächst meiotisch gefolgt von mitotischen Teilungen. Nach der doppelten Befruchtung bildet sich aus den Gameten sowohl der diploide Embryo, welcher die neue sporophytische Generation darstellt, als auch das triploide Endosperm.

Eine Reihe neuer zellulärer Schicksale wird während der Sporogenese, der Gametogenese und der Embryonalentwicklung begründet. Wachsende Evidenz belegt, dass nach der Meiose und während der Entwicklung der Gametophyten eine Neuprogrammierung des Chromatins stattfindet, die wahrscheinlich zur Differenzierung der Zellen beiträgt. Demgegenüber ist bislang unbekannt, ob Neuprogrammierungen des Chromatins auch dem Übergang von somatischem zu reproduktivem Schicksal unterliegen. Molekulare Studien legen nahe, dass eine epigenetische Reprogrammierung zur Spezifizierung der MMZ beiträgt. Diese beruht auf DNA Methylierungen und Histone Modifizierungen basierend auf der Aktivität von kleinen RNAs. Daher ist es möglich, dass weitere epigenetische Vorgänge am Übergang von somatischem zu reproduktivem Schicksal beteiligt sein könnten.

Wir haben eine robuste Methode entwickelt, um die Organisation des Zellkerns und die quantitative Verteilung der Modifizierungen des Chromatins mit hoher Auflösung in einzelnen MMZ in whole-mount eingebetteten *Arabidopsis* Ovulen zu untersuchen. Bemerkenswerter Weise haben wir gefunden, dass die Differenzierung der MMZ durch eine umfassende Neuprogrammierung des Chromatins begleitet wird. Diese umfassen eine Dekondensierung des Chromatins, eine Abnahme des Heterochromatins, einen Verlust der Linker Histone, Veränderungen der grundlegenden Histon-Varianten und Histon Modifizierungen. Das legt nahe, dass in den MMZ ein spezifisches Chromatin etabliert wird,

was bestimmte Veränderungen der epigenetischen und transkriptionellen Landschaft bedingt. Die Neuprogrammierung des Chromatins trägt wahrscheinlich dazu bei, Kompetenz für die postmeiotische Entwicklung und Gametopyhten-Entwicklung zu erwerben. Dies wurde auch durch die Analyse von Mutanten mit veränderter gametophytischer Kompetenz unterstützt.

Die umfangreiche Dynamik des Chromatins in der MMZ, die wir charakterisiert haben, weist auf die faszinierende Möglichkeit hin, dass das Epigenom vor der Meiose auf einen Grundzustand zurückgesetzt werden könnte, um die pluripotente Entwicklung der reproduktiven Linie zu ermöglichen. Um dieser Frage nachzugehen, haben wir eine genomweite Untersuchung von Chromatin Modifikationen in *Arabidopsis* MMZ angestrebt. Als Strategie haben wir eine spezifische Zellsortierung und Chromatin-Immunopräzipitation, gefolgt von einer genomweiten Sequenzierung („deep-sequencing“, ChiP-seq) gewählt. Die Strategie und vorbereitende Tests mittels INTACT System und Fluoreszenz Assistierter Zellsortierung werden präsentiert.

Abschließend haben wir angestrebt, die Frage zu beantworten, ob unsere Resultate spezifisch für das von uns untersuchte System (*Arabidopsis* MMZ) sind, oder ob sie eine breitere funktionale Relevanz haben. Unterliegt eine Neuprogrammierung des Chromatins auch dem Übergang von somatischem zu reproduktivem Schicksal in der männlichen Linie? Trifft dasselbe für andere Pflanzenarten zu? Um diesen Fragen nachzugehen, haben wir mit der Analyse von Zellkernorganisation und Chromatin Modifizierungen in der MMZ einer monokotyledonen Pflanze (Reis, *Oryza sativa*) und in der PMZ in *Arabidopsis* begonnen. Die vorläufigen Ergebnisse legen in der Tat nahe, dass eine Neuprogrammierung des Chromatins einen sich wiederholenden Prozess darstellen könnte, der sowohl dem männlichen als auch dem weiblichen Übergang von somatischem zu reproduktivem Schicksal in Blütenpflanzen unterliegt.

Abstract

The life cycle of flowering plants is marked by an alternation between a diploid sporophyte generation and a haploid gametophyte generation. The gametophyte generation is initiated by specification of spore mother cells (SMCs) in adult plant during flower development, which is different from that in animals where the germline is determined early during embryogenesis. The female SMC, or megaspore mother cell (MMC), derives from a subepidermal nucellar cell in the ovule primordium, while the male SMC, or pollen mother cell (PMC), differentiates from sporogenous tissue in the anthers. Both MMC and PMC specification marks the somatic-to-reproductive cell fate transition. The SMCs undergo meiosis followed by mitosis to form a multicellular, haploid gametophyte in which the gametes will differentiate. They will give rise to the diploid embryo, the new sporophytic generation, and the triploid endosperm following double fertilization.

Several new cell fates are established during sporogenesis, gametogenesis and embryogenesis. A growing body of evidence demonstrates that chromatin reprogramming events operate post-meiotically, during gametophyte development and embryo development, and likely contribute to cell differentiation. However, whether chromatin reprogramming also underlies the somatic-to-reproductive fate transition in the SMCs remains elusive. Genetic evidence implied that epigenetic reprogramming of the nucellar cells via small-RNA mediated DNA methylation and histone modifications contributes to MMC specification, suggesting further epigenetic events are possibly involved in the somatic-to-reproductive fate transition.

We developed a robust method to analyze nuclear organization and the quantitative distribution of chromatin modifications at high resolution in single MMCs, in whole-mount embedded *Arabidopsis thaliana* ovules. Notably, we found that MMC differentiation is accompanied by large-scale chromatin reprogramming, with chromatin decondensation, decrease of heterochromatin content, eviction of linker histones, changes of core histone variants and histone modifications, indicating the establishment of specific chromatin, which entails distinct epigenetic and transcriptional landscape, in MMCs. Chromatin reprogramming is likely to contribute to establishing competence for postmeiotic and gametophyte development, as evidenced by the analysis of mutants where the gametophytic competence was altered.

Extensive chromatin dynamics characterized in MMCs raised the fascinating possibility that the epigenome may be reset to a ground state before meiosis to enable pluripotent

development in the reproductive lineage. To address this question, we aimed at profiling the genome-wide distribution of chromatin modifications in *Arabidopsis* MMCs using a cell-specific sorting strategy and chromatin immunoprecipitation, followed by deep sequencing (ChIP-seq). The strategy and preliminary test using INTACT system and fluorescence assisted cell sorting are presented.

Finally, we aimed to answer the question whether our findings are specific to our system of study (the *Arabidopsis* MMC) or share a broader functional relevance. Does chromatin reprogramming also underlie the somatic-to-reproductive fate transition in the male lineage? In other plant species? To resolve this issue, we initiated the analysis of nuclear organization and chromatin modifications in the MMC of a monocot plant (rice, *Oryza sativa*) and in *Arabidopsis* PMC. Preliminary results indeed suggest that chromatin reprogramming may be a reiterated process underlying both male and female somatic-to-reproductive fate transition among flowering plants.

1. General Introduction

1.1 Sexual reproduction in flowering plants

The life cycle of flowering plants alternates between a predominant diploid sporophytic phase and a haploid gametophytic phase. The process of sexual reproduction in flowering plants is marked by several waves of cell fate transitions: from the somatic to reproductive cell fate during sporogenesis, from the haploid spores to the gametes during gametogenesis, and from the gametes to the embryo and endosperm following double fertilization (Figure 1).

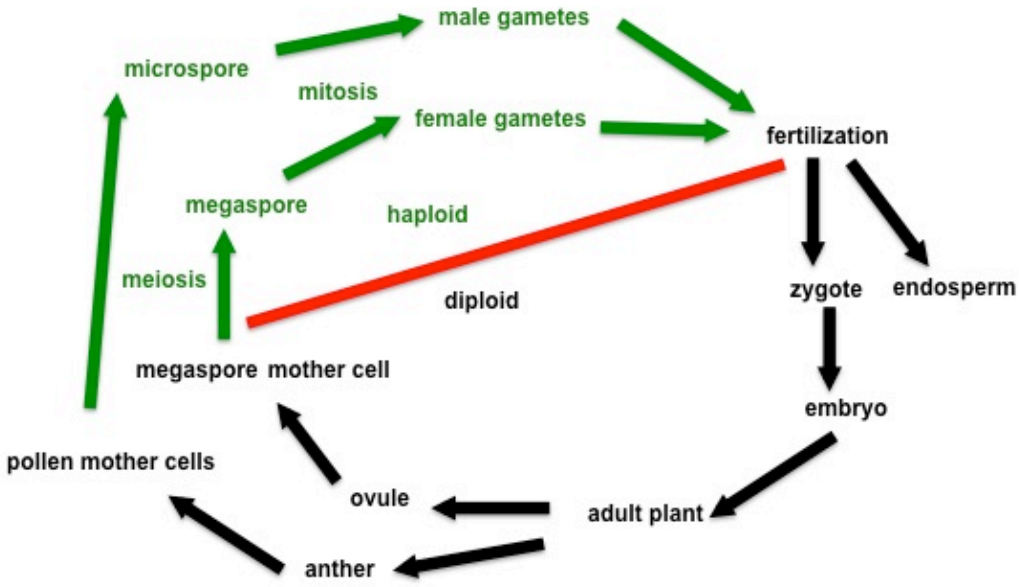

Figure 1. Life cycle of flowering plants, with an alternation between a diploid sporophytic generation and a haploid gametophyte generation. The diploid phase is marked by the vegetative development from the embryo to the adult plant, and differentiation of spore mother cells in the reproductive organs. The haploid phase is initiated by meiotic division of diploid spore mother cells and followed by mitotic divisions to form haploid gametes.

1.1.1 Sporogenesis in flowering plants

Sexual reproduction in flowering plants begins with sporogenesis where spore mother cells (SMCs) differentiate from somatic cells in the floral organs of adult plants (Figure 2, Maheshwari, 1950). Female SMCs, also called megaspore mother cells (MMCs) differentiate within ovule primordia in the gynoecium. In *Arabidopsis*, a single sub-epidermal cell at the distal end of each ovule pridormium enlarges to form the archesporial cell, which in turn

directly develops into MMC (Figure 2, Maheshwari, 1950). While this process varies among species, which is displayed by the existence of several MMCs in each ovule primordium or formation of MMC indirectly from the divided archesporial cell (Maheshwari, 1950). MMC then undergoes meiosis to produce four haploid spores while only one survives to form the functional megaspore (Figure 2). Male SMCs, also called pollen mother cells (PMCs) or microspore mother cells differentiate within the sporangium formed in the anther locule. In *Arabidopsis*, one hypodermal cell in the sporangium enlarges to form the archesporial cell which then divides to generate the primary sporogenous cell towards the inside and primary parietal cell in the outside, the sporogenous cell undergoes mitosis to give rise to PMCs, while the primary parietal cell forms several layers of walls surrounding PMCs via periclinal and anticlinal divisions comprising of epidermis, endothecium, middle layers and tapetum (Figure 3, Maheshwari, 1950). Each PMC then undergoes meiosis to generate four haploid microspores (Figure 4). Thus, unlike that in animals where the germline lineage is established early in embryogenesis, there is no predetermined germline lineage in flowering plants. Instead, SMC differentiation marks a somatic-to-reproductive cell fate transition in flowering plants, where the reproductive lineage encompasses here the SMC until the mature gamete.

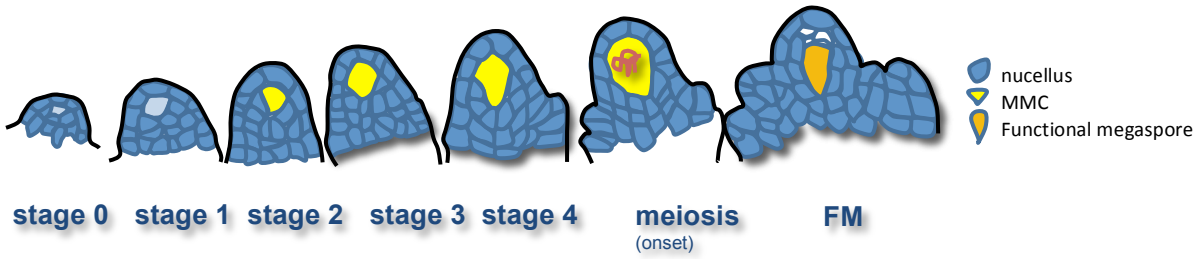

Figure 2. Megasporogenesis in flowering plants. The sub-epidermal nucellar cell enlarges to differentiate into megaspore mother cell (MMC), which then undergoes meiosis to give rise to the functional megaspore (FM). This figure was drawn by Célia Baroux (University of Zürich).

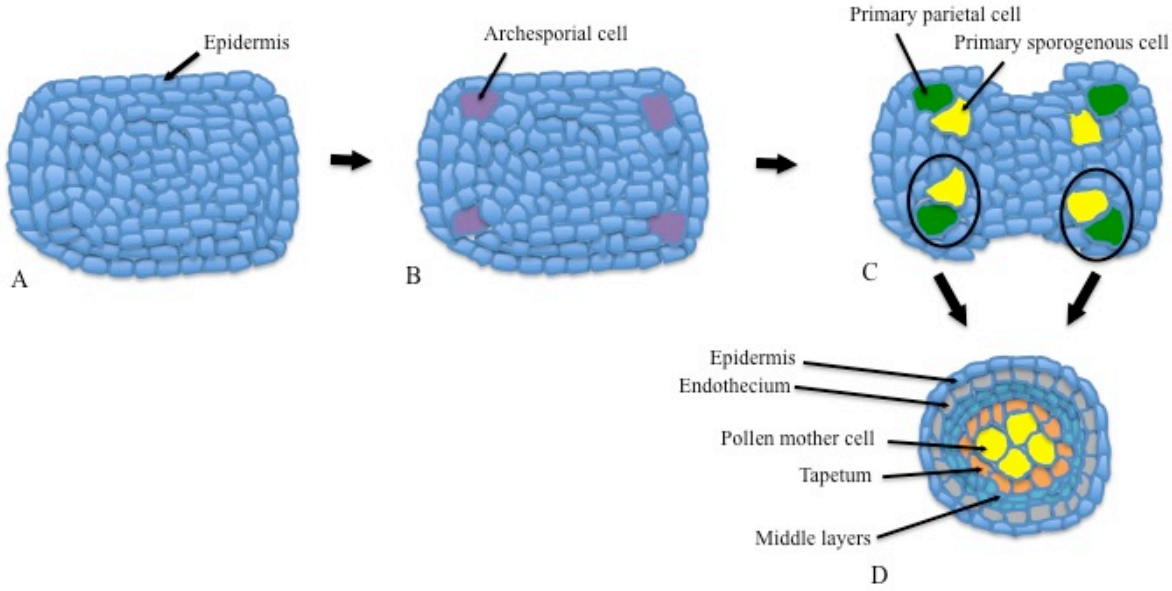

Figure 3. Development of anther (microsporangium) with differentiation of pollen mother cells (PMCs, or microspore mother cells) is shown in transverse section. (A) Stamen primordium is a homogeneous mass of meristematic cells surrounded by epidermis. (B) One subepidermal cell in each of the four corners of stamen primordium enlarges to form the archesporial cell. (C) Each of the archesporial cells then divides to give rise to a primary sporogenous cell on the inner side and a parietal cell towards the outside. (D) The parietal cell divides periclinally and anticlinally to generate the wall of the anther, comprising of epidermis, endothecium, middle layers and tapetum, while the sporogenous cell divides to give rise to a number of microspore mother cells or pollen mother cells.

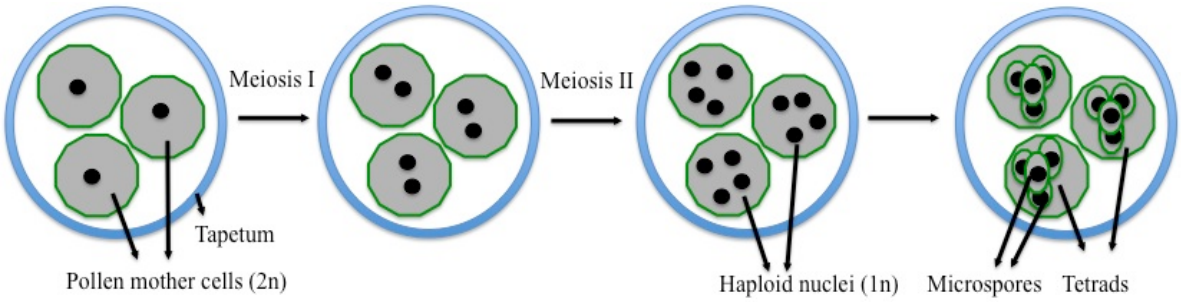

Figure 4. Formation of micropsores in the anther. Each pollen mother cell undergoes meiosis to gives rise to four haploid nuclei, these nuclei are arranged as a tetrad and soon surrounded by cell walls, which are then called the microspores.

1.1.2 Gametogenesis in flowering plants

During gametogenesis, the functional megaspore undergoes three rounds of mitosis to give rise to the female gametophyte harboring two gametes, the egg cell and the central cell, as well as five accessory cells (Figure 5A). While, microgametogenesis begins with an asymmetric mitosis in each microspore, resulting in formation of a larger vegetative cell and a smaller generative cell within the bicellular pollen grain. The vegetative cell arrests at G1-phase and later attenuates differentiation, while the generative cell undergoes another round of mitosis to produce the gametes: two sperm cells harboring identical genetic information (Figure 5B) (McCormick, 1993; Drews and Yadegari, 2002; Yadegari and Drews, 2004).

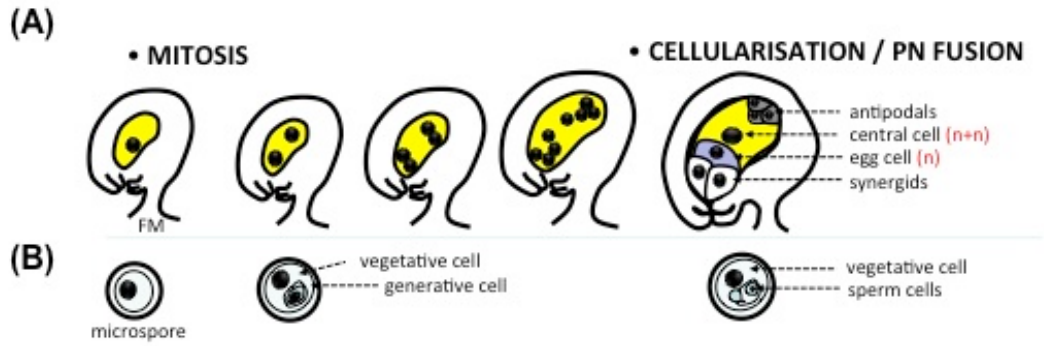

Figure 5. Gametogenesis during plant sexual reproduction. (A) The functional megaspore (FM) undergoes three rounds of mitosis and cellularization to give rise to the female gametophyte (embryo sac), consisting of two gametes: the egg cell and the central cell, accompanied with three antipodals and two synergids. (B) The microspore undergoes mitosis to produce one vegetative cell and one generative cell, while the generative cell divides further to give rise to the gametes: two sperm cells in the male gametophyte (pollen). This was modified from C. Baroux (University of Zürich).

1.1.3 Double fertilization in flowering plants

During double fertilization, the egg cell fuses with one sperm to produce the diploid zygote that will give rise to the embryo, while the central cell is fertilized by another sperm to generate the triploid endosperm. Thus, cell fates are transformed from the haploid gametes to the diploid zygote and triploid endosperm by uniting the parental genome (Figure 6, Maheshwari, 1950).

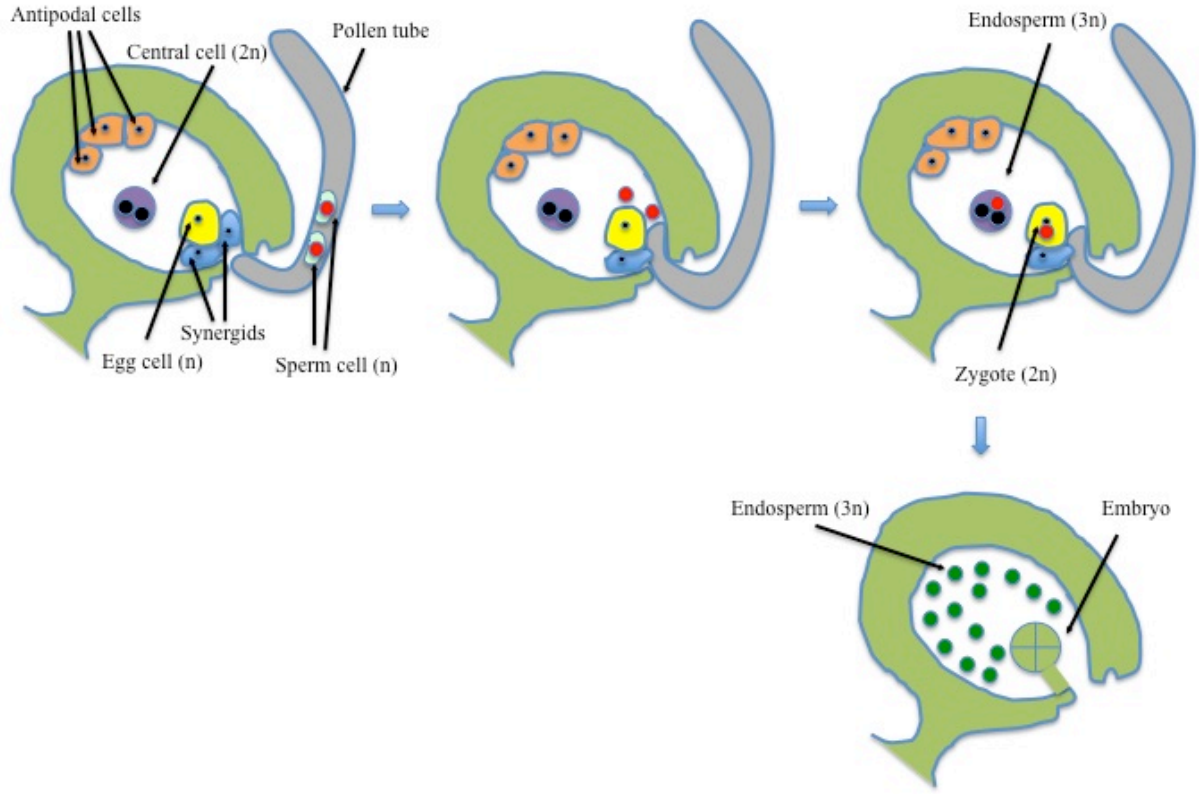

Figure 6. Double fertilization in flowering plants. The egg and central cell are fused with one sperm to give rise to the diploid zygote that will form the embryo and triploid endosperm respectively.

1.2 Chromatin organization and modification in plants

1.2.1 Interphase chromatin organization in plants

In multicellular organisms, somatic cells harboring identical genetic material may dramatically change their transcriptional patterns and thus their functions in response to environmental or developmental signals, which in turn acquire new cell fates and cell identity. Genome expression is often regulated by chromatin structure. The basic building block of chromatin is nucleosome, which is composed of approximately 147bp of DNA wrapped in 1.65 superhelical turns around an octamer particle (containing two molecules of each of the core histones H2A, H2B, H3 and H4) (Luger, 1997). The nucleosome is separated from each other by linker DNA binding with linker histone H1 at the entry-exit point, which facilitates chromatin compaction (Hood and Galas, 2003). The nucleosomes are not simply arranged as a string, but self-organized into higher-order chromatin structures.

Similar to that in other eukaryotes, chromatin in plants exists as two forms: euchromatin and heterochromatin. Euchromatin appears as loosely coiled DNA that is dispersed, and allows for an open, transcriptionally permissive state, while heterochromatin is tightly packed, that usually entails a compact, repressive state (Franklin and Cande, 1999; Fransz, 2002). Heterochromatin comes in two varieties: facultative heterochromatin and constitutive heterochromatin. The facultative heterochromatin is confined to specific cell types, and can turn to a decondensed state that allows active transcription under certain developmental or environmental cues. While the most common type, constitutive heterochromatin, refers to the region that is rich in repetitive DNA, and invariably transcriptionally inert, which usually occurs around centromeres and subtelomeric nucleolar organizing regions (NORs). The centromere core is mainly composed of 180-by tandem repeat and transposon-like sequences. While NORs are characterized by tandem repeats of ribosomal genes such as 45S rDNA repeats (Fransz et al., 2002; Fransz and de Jong, 2011).

In plant species with large-sized genome, such as maize and barley, RabI configuration is adopted in the interphase nucleus, with polarized localization of centromeres and telomeres, (Cowan et al., 2001; Fransz et al., 2002; Fransz and de Jong, 2011; Tiang et al., 2012) (Figure 7A). While in those species with small genomes, such as *Arabidopsis*, constitutive heterochromatin is organized as conspicuous chromocenters (CCs) that harbor centromeric, pericentromeric repeats during interphase, that are peripherally located in the nucleus, which are subject to repressive epigenetic modifications such as DNA methylation and mono/di H3K9 methylation (Fransz et al., 2002; Fransz et al., 2006; Fransz and de Jong, 2011). From chromocenters, the gene-rich euchromatic loops emanate to form a rosette-like structure (Figure 7B). The subtelomeric nucleolar organizing region (NOR) may serve as the basis of the chromatin loop. Chromocenters and the loops together form the chromosome territories (CTs), with side-by-side arrangement of heterologous CTs and association of homologous CTs in a random frequency, except for NOR-bearing chromosomes which are paired at higher frequency (Fransz et al., 2002; Pecinka et al., 2004; van Driel and Fransz, 2004; Berr and Schubert, 2007; Fransz and de Jong, 2011; Tiang et al., 2012). CTs interaction can be supported by a recent study using chromosome conformation capture, which demonstrated that *cis* interactions occur within chromosome arms, with distinguished interactomes formed between heterochromatin and euchromatin. However, heterochromatin islands can partially evade to the surrounding euchromatin. The heterochromatic and euchromatic interactomes are marked by and most probably influenced by their distinct epigenetic landscapes (Grob et al., 2013).

Chromatin can be modulated by multiple epigenetic modifications, including covalent histone modifications, DNA methylation, incorporation of histone variants, noncoding RNA, as well as ATP-dependent chromatin remodeling, which influence the accessibility of molecular factors such as transcriptional factors to DNA (Figure 8) (Jenuwein and Allis, 2001; Liu et al., 2010; Grimanelli and Roudier, 2013). Thus, dynamic change of chromatin structure influences genome expression, which in turn contributes to cellular function. For a long time, investigations of chromatin dynamics accompanying plant cell differentiation had been impaired, mostly because of technical difficulties in applying cytogenetic tools to cells *in planta*. Recent progresses in the methodologies, or the choice of cell culture systems, renewed the interest in this field. Reports of chromatin and chromosome changes upon de- and re-differentiation in cell culture (Williams et al., 2003; Tessadori et al., 2007), root differentiation (Costa and Shaw, 2006), or endosperm development (Wegel and Shaw, 2005; Wegel et al., 2005; Baroux et al., 2007a) indicate that the plant chromatin is extremely dynamic.

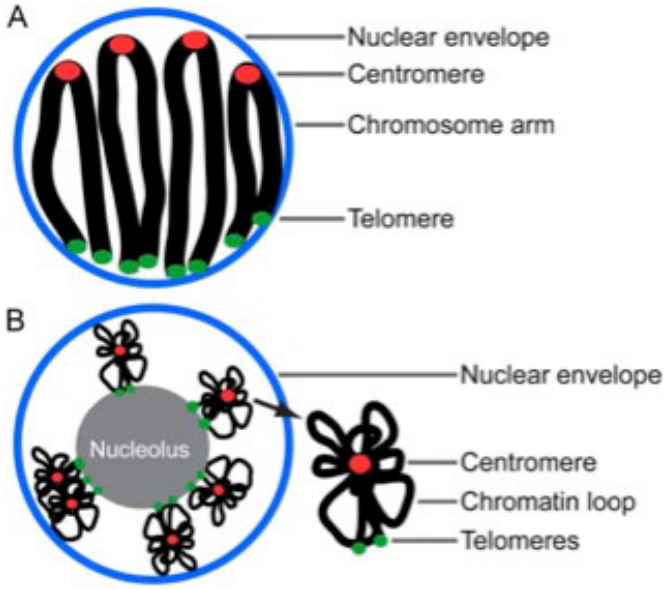

Figure 7. Chromatin arrangement in the interphase nucleus of plants. A, Rabl configuration exists in interphase nuclei of many large-genome plant species. B, Rosette-like organization of chromosomes in interphase nuclei of *Arabidopsis*. This figure was from Tiang et al., 2012.

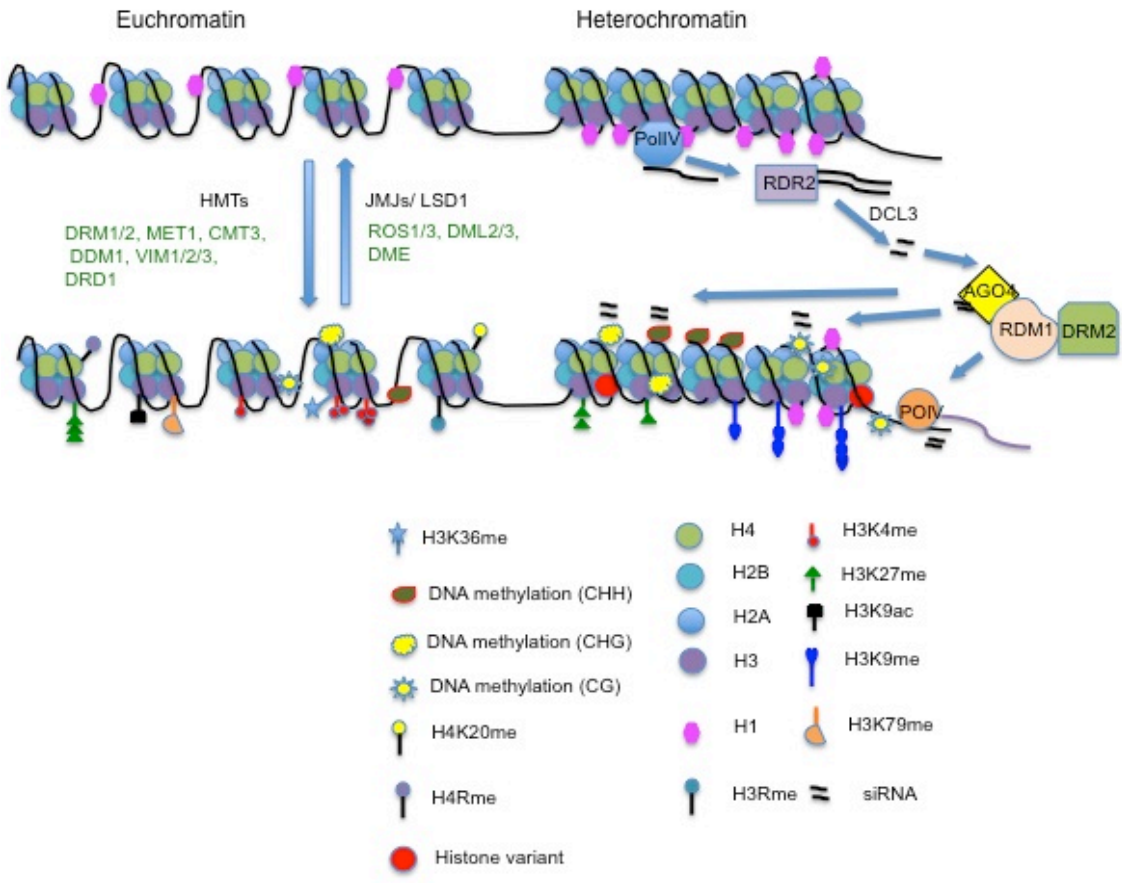

Figure 8. Modulation of chromatin structure via histone modification, incorporation of histone variants, DNA methylation, and small RNA-directed DNA methylation (RdDM) in plants. N-terminal tails of core histones can be subject to various posttranslational modifications, with methylation on the lysine (Lys, K) and arginine (Arg, R) residues of histone H3 and H4 as the most commonly modifications, which are methylated by histone methyltransferases (HMTs) and demethylated by JMJs or LSD1 proteins. In plants, DNA methylation occurs in CG, CHG and CHH contexts, which are established by DRM1/2. CG methylation is maintained by MET1, and controlled by VIM1/2/3, DDM1 and the chromatin remodeller DRD1, while CHG is methylated by CMT3. DNA methylation is not static, the methyl groups can be removed by the glycosylase proteins ROS1/3, DME, DML2, and DML3. Chromatin structure can be also regulated by small RNA directed DNA methylation (RdDM pathway). The PolIV transcripts ssRNA is converted by RDR2 into dsRNA, which is then processed into siRNAs by DCL3. siRNAs are then loaded onto AGO4 to guide DNA methylation at all sequence contexts, which is dependent on recruitment of the de novo DNA methyltransferase (DRM2).

1.2.2 Histone post-translational modifications in plants

Chromatin structure can be modulated by various posttranslational modifications on N-terminal tails of core histones, including methylation, acetylation, ubiquitination, phosphorylation, glycosylation, and sumoylation, thereby regulating accessibility of factors for DNA-templated processes (Strahl and Allis, 2000). A hypothesized "histone code" was generated by the combinatory role of different histone modifications, which allows for fine-tuning of gene expression. Compared to that in other organisms, plants entail both conserved and unique histone modifications (Johnson et al., 2004; Zhang et al., 2007a).

1.2.2.1 Histone methylation by HMTs in plants

In plants, histone methylation occurs mainly on the lysine (Lys, K) and arginine (Arg, R) residues of histone H3 and H4. The lysine can be mono-, di- or trimethylated at K4, K9, K27, K36 and K79 of histone H3, as well as K20 of histone H4, while arginine methylation is restricted to mono-, or dimethylation at R2, R17, and R26 of histone H3, as well as R3 of H4, which is catalyzed by arginine HMTs (Figure 8) (Nelissen et al., 2007). Histone lysine methylation is exerted by histone methyltransferases (HMTs), the SET Domain Group (SDG) proteins, except that H3K79 is methylated by the non-SET domain-containing lysine HMTs. The evolutionarily conserved SET catalytic domain is derived from the initially identified Drosophila HMTs Suppressor of variegation (Su(var)3-9), Enhancer of Zeste (E(z)) and Trithorax (TRX). *Arabidopsis*, rice and maize genomes encode at least 47, 38 and 36 SDG proteins respectively, which can be assigned into four distinct subgroups: SU(VAR)3–9 groups [including SU(VAR)3–9 homologs (SUVH) and SU(VAR)3–9 related proteins (SUVR)], E(Z) homologs, TRX groups (TRX homologs and TRX-related proteins), and ASH1 homologs related proteins (Baumbusch et al., 2001; Springer et al., 2003; Nelissen et al., 2007; Ng et al., 2007; Gendler et al., 2008; Liu et al., 2010; Punta et al., 2012).

Trithorax group (TrxG) proteins are responsible for H3K4 and H3K36 methylation that mark active transcription. ARABIDOPSIS TRITHORAX RELATED3 (ATXR3/SDG2) is the major H3K4 trimethyltransferase, which mediates global genome-wide H3K4me3 deposition. Its loss of function causes pleiotropic defects in both sporophytic and gametophytic development (Berr et al., 2010; Guo et al., 2010). While, ARABIDOPSIS TRITHORAX1 (ATX1/SDG27) is required for active transcription of FLOWERING LOCUS C (FLC) and flower homeotic genes via deposition of H3K4me3, thereby regulating flowering time and

flower organ identity (Alvarez-Venegas et al., 2003; Pien et al., 2008). ATX2/SDG30 was shown to be involved in H3K4me2 deposition, albeit its depletion causes no obvious phenotype (Pien et al., 2008; Saleh et al., 2008). ASHR3/SDG4 operates stamen and pollen development through regulating H3K4me2 and H3K36me3 deposition (Cartagena et al., 2008; Thorstensen et al., 2008). ASHH2/SDG8 is the major H3K36 di/trimethyltransferase, which is required for controlling flowering time by activation of FLC via H3K36 di-/trimethylation, regulating shoot branching and carotenoid composition, its depletion leads to multiple defects such as early flowering, reduced organ size, as well as abnormal carotenoid composition (Zhao et al., 2005; Xu et al., 2008; Cazzonelli et al., 2009). In addition, H3K4 and H3K36 methylation can be slightly affected by ATXR7/SDG25 in vitro (Berr et al., 2009; Tamada et al., 2009).

H3K9 methylation is mediated by the SU(VAR)3–9 histone methyltransferase, which control the establishment of heterochromatin domains in eukaryotes. In plants, H3K9 is predominately mono- or dimethylated, which is enriched in chromocenters, retroelements and repetitive sequences, whereas H3K9 trimethylation mainly exists as a euchromatin mark, but also marks transposons and pseudogenes at low frequency (Johnson et al., 2004; Charron et al., 2009; Veiseth et al., 2011). In *Arabidopsis*, 10 *SUVH* genes encode SU(VAR)3-9 homologues (SUVH) (Baumbusch et al., 2001; Naumann et al., 2005). KRYPTONITE (KYP/SUVH4) was the first identified H3K9 methyltransferase in plant, which was characterized in a mutant screen for suppressors of gene silencing at the *Arabidopsis thaliana* SUPERMAN (SUP) locus. H3K9 mono- or dimethylation catalyzed by KYP orchestrates CpNG DNA methylation and retrotransposon silencing via interaction with the DNA methyltransferase CMT3 (CHROMOMETHYLASE3) (Jackson et al., 2002). It is noteworthy that SUVH2 also plays central roles in heterochromatin formation and silencing through interaction with MET1 and DDM1, with its loss of function resulting in significant reduction of all heterochromatic histone methylation marks including H3K9me1, H3K9me2, H3K27me1, H3K27me2, and H4K20me1 (Naumann et al., 2005; Fischer et al., 2006). Additionally, SUVH5 and SUVH6 were also shown to be involved in H3K9 mono- or dimethylation, with the triple mutant *suvh4suvh5suvh6* losing H3K9 mono/di methylation at target loci and reduction of non-CG methylation phenocoping *cmt3* mutation, suggesting that CMT3 activity is controlled by SUVH4, SUVH5 and SUVH6 (Jackson et al., 2004; Ebbs et al., 2005; Ebbs and Bender, 2006). In addition to these active *SUVH* genes, there are five genes (*SUVR1-5*) encoding SU(VAR)3-9 related (SUVR) proteins in *Arabidopsis*, with most of them localized in the nucleolus or nuclear bodies (Baumbusch et al., 2001; Thorstensen et

al., 2006). SUVR4 is involved in converting H3K9me1 to H3K9me3 through binding with ubiquitin only on transposon chromatin, but not euchromatin, thus contributing to genome integrity via repressing transposon activity (Veiseth et al., 2011).

Unlike that for H3K9 and H3K4 methylation, enzymes catalyzing heterochromatic H3K27 monomethylation remains largely unknown. The SET-domain proteins ARABIDOPSIS TRITHORAX-RELATED PROTEIN5 (ATXR5) and ATXR6 are the only characterized methyltransferases which are responsible for H3K27me1 deposition, mutations in *atxr5atxr6* cause reduced H3K27me1 in vivo and partial heterochromatin decondensation accompanied by activation of repressed DNA repeats and transposons in chromocenters, but without DNA methylation and H3K9me2 affected, suggesting a distinct pathway for regulating chromatin structure and transcriptional silencing (Jacob et al., 2009). By contrast to H3K27 monomethylation, the repressive mark H3K27me3 is localized in euchromatin in plants, which functions in different developmental phases via repressing target genes (Turck et al., 2007). In *Drosophila melanogaster*, Polycomb Repressive Complex 2 (PRC2) comprising Extra sex comb (ESC), Enhancer of zeste (E(Z)), Supressor of zeste 12 (SU(Z)12), and p55 acts to catalyze tri-methylation of H3K27 in vivo through interaction of E(Z) and Esc (Müller et al., 2002; Ringrose and Paro, 2004; Ketel et al., 2005). Homologs of all members of the conserved PRC2 complex are present in *Arabidopsis*, including three E(Z) homologs [CURLY LEAF (CLF), MEDEA (MEA), and SWINGER (SWN)], three Su(z)12 homologs [FERTILIZATION-INDEPENDENT SEED2 (FIS2), EMBRYONIC FLOWER2 (EMF2), and VERNALIZATION2 (VRN2)], five p55 homologs [MULTICOPY SUPPRESSOR OF IRA (MSI)1–5], and only one homolog of Esc [FERTILIZATION-INDEPENDENT ENDOSPERM (FIE)] (Pien and Grossniklaus, 2007; Liu et al., 2010). Although lacking direct biochemical evidence, the E(Z) homologs including CLF, MEA, and SWN are widely believed to catalyze H3K27 trimethylation via formation of the PRC2 complex in *Arabidopsis*. At least three distinct PRC2 complexes are identified to regulate plant development. The FIS-PRC2 complex, which is composed of MEA, FIS2, FIE and MSI1, is required for female gametophyte and seed development (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999; Köhler et al., 2003; Pien and Grossniklaus, 2007; Bemer and Grossniklaus, 2012). The EMF2-PRC2 complex, consisting of EMF2, CLF, FIE, and MSI1, is central in repressing floral transition and floral homeotic gene transcription. In *emf2* mutnat, H3K27me3 at the floral organ identity gene *AGAMOUS* (*AG*) is dramatically decreased, which is accompanied by ectopical activation of *AG* and some other floral organ identity genes including *APETALA* (*AP1*), *AP3*, and *PISTILLATA* (*PI*) (Moon, 2003; Calonje et al.,

2008; Liu et al., 2010). EMF2, CLF, and FIE are involved in silencing the floral repressor FLC and the florigen gene *FLOWERING LOCUST (FT*), with lost of H3K27me3 at AG, AGL19 (*AGAMOUS LIKE19)*, FLC, as well as *FT* in *clf* mutants, resulting in early flowering, curled leaves and aberrations of floral organs (Jiang et al., 2008; Liu et al., 2010). Additionally, SWN was shown to act redundantly with MEA and CLF, with stronger effects in *swnmea* and *swnclf* double mutants, and decrease of H3K27me2/3 in *swnclf* double mutant (Chanvivattana et al., 2004; Makarevich et al., 2006; Wang et al., 2006; Liu et al., 2010; Lindroth et al., 2011). The VRN2-PRC2 complex, comprised by SWN/CLF, VRN2, FIE and MSI1, regulates the spreading of H3K27me3 across the FLC loci to maintain repressed state of FLC during vernalization (Bastow et al., 2004; Finnegan and Dennis, 2007; De Lucia et al., 2008). While, the recruitment of LIKE HETEOCHROMATIN PROTEIN 1 (LHPI), also named as TERMINAL FLOWER 2 (TFL2), by H3K27me3 contributes to FLC repression (Gaudin et al., 2001; Mylne et al., 2006; Sung et al., 2006; Turck et al., 2007; Zhang et al., 2007b). Chromatin modifiers that involved in histone methylation in *Arabidopsis* are listed in Table 1.

1.2.2.2 Histone demethylation in plants

Histone methylation is a dynamic process, which can be reversed by histone demethylase (Figure 8). It is known that two types of demethylases, namely lysine-specific demethylase1 (LSD1) and Jumonji C (JmjC) domain-containing proteins (JMJs), are employed to remove the methyl groups from different methylated substrates which is dependent on distinct mechanisms and cofactors (Shi et al., 2004; Tsukada et al., 2006; Klose and Zhang, 2007). There are four LSD1 homologs present in *Arabidopsis*, including LSD1-LIKE 1(LDL1), LDL2, LDL3 and FLOWERING LOCUS D (FLD). LDL1 is shown to mediate H3K4 mono- and di-demethylation. LDL1, LDL2 and FLD are required for flowering time control, with elevation of H3K4me2 at FLC locus in *ldl1ldl2* and *ldl1fld* double mutants, while LDL1 and LDL2 can affect H3K4me2 independently (Jiang et al., 2007). *Arabidopsis* genome encodes at least 21 JMJ-domain proteins, with five of them showing demethylase activity. JMJ14 and JMJ15 are both required for H3K4me1/2/3 demethylation. JMJ14 acts downstream from the Argonaute effector complex to demethylate H3K4 at the target of RNA silencing, with its mutation causing early flowering, reduction of non-CG methylation, and defects in RNA silencing (Deleris et al., 2010; Lu et al., 2010; Searle et al., 2010). Mutants lacking JMJ15 activity cause no obvious phenotype, whereas overexpression of JMJ15 leads to early

flowering via demethylation of H3K4me3 at FLC locus (Yang et al., 2012). *Relative of Early Flowering 6* (*REF6*/*JMJ12*) was shown to specifically demethylate H3K27me2/3, with ectopic increase of H3K27me3 and repression of hundreds of genes related to developmental patterning and stimuli response in *ref6* mutant (Lu et al., 2011). Interestingly, loss of function of *Early Flowering 6* (*ELF6*/*JMJ11)* and its homolog *REF6* cause early flowering and late flowering respectively, with downregulation of brassinosteroid-regulated genes and elevation of H3K9me3, indicating that they may act as H3K9 demethylase (Noh et al., 2004; Yu et al., 2008). In addition, Increase in Bonsai Methylation 1 (IBM1/JMJ25) was reported as H3K9 demethylase for protection of active genes against heterochromatinization (Saze et al., 2008; Miura et al., 2009; Inagaki et al., 2010; Berr et al., 2011). Chromatin modifiers that involved in histone demethylation in *Arabidopsis* are listed in Table 1.

Table 1. Known chromatin modifers for methylation and demethylation of histones in *Arabidopsis*.

Chromatin modifiers	Enzyme	Targets	References
Histone methyltransferases SET Domain Group			
Trithorax group (TrxG)	ATXR3/SDG2	H3K4me3	(Berr et al., 2010; Guo et al., 2010)
	ATX1/SDG27	H3K4me3	(Alvarez-Venegas et al., 2003; Pien et al., 2008)
	ATX2/SDG30	H3K4me2	(Pien et al., 2008; Saleh et al., 2008)
	ASHR3/SDG4	H3K4me2 H3K36me3	(Cartagena et al., 2008; Thorstensen et al., 2008)
	ASHH2/SDG8	H3K36me2/3	(Zhao et al., 2005; Xu et al., 2008; Cazzonelli et al., 2009)
	ATXR7/SDG25	H3K4me H3K36me	(Berr et al., 2009; Tamada et al., 2009)
	ATXR5	H3K27me1	(Jacob et al., 2009)
	ATXR6	H3K27me1	(Jacob et al., 2009)

E(Z) homologs	CLF, MEA, SWN	H3K27me3	(Chanvivattana et al., 2004; Makarevich et al., 2006; Wang et al., 2006; Liu et al., 2010; Lindroth et al., 2011)
SU(VAR)3–9 groups	KYP/SUVH4	H3K9me1/2	(Jackson et al., 2002)
	SUVH2	H3K9me1/2 H3K27me1/2 H4K20me1	(Naumann et al., 2005; Fischer et al., 2006)
	SUVH5	H3K9me1/2	(Jackson et al., 2004; Ebbs et al., 2005; Ebbs and Bender, 2006)
	SUVH6	H3K9me1/2	(Jackson et al., 2004; Ebbs et al., 2005; Ebbs and Bender, 2006)
	SUVR4	H3K9me3	(Veiseth et al., 2011)
Histone demethylases			
Lysine-Specific Demethylase1 (LSD1)	LDL1	H3K4me1/2	(Jiang et al., 2007)
	LDL2	H3K4me2	(Jiang et al., 2007)
	FLD	H3K4me2	(Jiang et al., 2007)
Jumonji C domain-containing proteins (JMJs)	JMJ14	H3K4me1/2/3	(Deleris et al., 2010; Lu et al., 2010; Searle et al., 2010)
	JMJ15	H3K4me1/2/3	(Yang et al., 2012)
	REF6/JMJ12	H3K27me2/3 H3K9me3	(Yu et al., 2008; Lu et al., 2010)
	ELF6/JMJ11	H3K9me3	(Noh et al., 2004; Yu et al., 2008)
	IBM1/JMJ25	H3K9	(Saze et al., 2008; Miura et al., 2009; Inagaki et al., 2010; Berr et al., 2011)

1.2.3 DNA methylation and demethylation in plants

DNA cytosine methylation (5mC) is widespread in most of the eukaryotes, which is a prominent epigenetic modification and implicated in the silencing of transposon elements (TEs) and endogeneous genes. Unlike that in animals, where most DNA methylation is restricted to CG dinucleotides, cytosine methylation in higher plants covers all three sequence contexts: CG, CHG and CHH (H represents A, C or T, Figure 8) (Chan et al., 2005). High levels of DNA methylation are enriched on transposons, retrotransposons, rDNA arrays and

centromeric repeats. Patterns of DNA methylation are established by *de novo* methyltransferases and retained by maintenance of methyltransferases. In *Arabidopsis*, DOMAINS REARRANGED METHYLTRANSFERASE 1and 2 (DRM1/2), the homologues of the mammalian Dnmt3 family members, are required for *de novo* DNA methylation in all three contexts via RNA-directed DNA methylation (RdDM) (Cao and Jacobsen, 2002). DNA METHYLTRANSFERASE1 (MET1), a plant homologue of Dnmt1, mediates the maintenance of CG methylation, and functions in the silencing of heterochromatic region (Kankel et al., 2003; Zhang et al., 2010). It was also reported to be potentially involved in the maintenance of non-CG methylation (Finnegan et al., 1998). CG methylation is also regulated by the chromatin remodeling ATPases DECREASE IN DNA METHYLATION 1 (DDM1), VARIANT IN METHYLATION 1, 2, 3 (VIM1, 2, 3) and the chromatin remodeling protein DRD1 (Jeddeloh et al., 1999; Kanno et al., 2005; Woo et al., 2008). Methylation in the CHG context is maintained by CHROMOMETHYLASE3 (CMT3) with a combinatory role of KYP mediated H3K9 methylation, which functions in the maintenance of epigenetic gene silencing (Bartee et al., 2001; Lindroth et al., 2001; Jackson et al., 2002). While maintenance of CHH methylation is dependent on DRM1 and DRM2 via RdDM pathway, and partly contributed by CMT3 (Lindroth et al., 2001; Cao and Jacobsen, 2002; Furner and Matzke, 2011).

DNA methylation is labile epigenetic mark and can be erased via active and passive demethylation pathways. Role of passive DNA demethylation can be evidenced by demethylation of DNA during female gametogenesis dependent on MET1 suppression via Retinoblastoma pathway. MET1 is repressed by RETINOBLASTOMA RELATED1 (RBR1) binded with MULTICOPY SUPRESSOR OF IRA1 (MSI1) during female gametogenesis, resulting in passive loss of DNA methylation, which in turn leads to the expression of maternal imprinted genes like *FIS2* and *FWA* (*FLOWERING OF WAGENINGEN*) (Jullien et al., 2008). While methylated marks can be also actively removed by DNA glycosylases including REPRESSOR OF SILENCING 1 (ROS1), ROS3, DEMETER (DME) and its homologues DEMETER LIKE 2 (DML2), DML3 (Choi et al., 2002; Gong et al., 2002; Ortega-Galisteo et al., 2008; Zheng et al., 2008). Expression of DME in the central cell during female gametogenesis is required for the activation of the maternal alles MEDEA, FIS2 and FWA, thus contributing to the expression of these maternal imprinted genes in the endosperm (Choi et al., 2002; Kinoshita et al., 2004; Gehring et al., 2006; Zhang et al., 2010). In addition, DME mediated TEs demethylation in the companion cells including the central cell and the vegetative cell reinforces TEs silencing in the gametes via sRNA-directed DNA methylation (Ibarra et al., 2012). By contrast to DME, ROS1 is widely expressed throughout

development, which is involved in transcriptional silencing, possibly guided by ROS3 bounded RNAs (Gong et al., 2002; Zheng et al., 2008). DML2 and DML3 are required not only for removal of methyl marks from improperly methylated cytosines, but also for maintenance of high levels of proper DNA methylation (Penterman et al., 2007; Ortega-Galisteo et al., 2008).

1.2.4 Small RNA pathways in plants

Small RNA pathways have emerged to be involved in epigenetic regulation of gene expression. In plants, small RNA can be grouped into two main classes: microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs originates from the loci that generate ssRNAs. A hairpin structure is then formed from ssRNAs, which in turn is processed into 20-24nt small RNAs by specific enzymes like DICER-LIKE 1 (DCL1). These miRNAs are then loaded onto a RNA-induced silencing complex (RISC) with the AGONAUTE family member AGO1 recruited, and directed to the target mRNA via base pairing. miRNAs are considered to play central roles in phase transition, hormone biosynthesis and signaling, pattern formation, and morphogenesis during plant development (Chen, 2009; Le Trionnaire and Twell, 2010; Axtell, 2013). While siRNAs are produced from the loci including noncoding RNAs, TEs or repeats that generates dsRNAs that is processed into 18-25nt siRNAs (Le Trionnaire and Twell, 2010). siRNA can be further subdivided into heterochromatic siRNAs, secondary siRNAs and natural antisense transcript siRNAs (NAT-siRNAs. Heterochromatic siRNAs derive from intergenic and/or repetitive genome regions, which are associated with de novo DNA methylation and H3K9 methylation at target loci (Axtell, 2013). ssRNA is generated by RNA Polymerase IV (PolIV), and converted into dsRNA via RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), which is then processed into siRNAs by DICER-LIKE 3 (DCL3), and stabilized by HUA ENHANCER 1 (HEN1) via 3'-terminal ribose methylation (Xie et al., 2004; Pikaard et al., 2008; Chen, 2009; Zhang and Zhu, 2011; Axtell, 2013). siRNAs are then loaded onto AGO4-clade AGOs to form the siRNA/AGO duplexes to guide DNA methylation by pairing with complementary DNA targets or nascent scaffold RNAs from the DNA targets. Here, the scaffold RNAs are generated from intergenic non-coding regions by PolII and PolV, which is dependent on the putative chromatin-remodeling protein DRD1 (DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1), the chromosome hinge domain protein DMS3 (DEFECTIVE MERISTEM SILENCING 3), and RDM1 (RNA DIRECTED DNA METHYLATION 1), and

possibly required for silencing adjacent siRNA-generating loci (Kanno et al., 2004; Kanno et al., 2008; Wierzbicki et al., 2008; Wierzbicki et al., 2009; Zheng et al., 2009). Furthermore, successful targeting also requires the recruitment of chromatin modifiers like DRM2 by heterochromatic siRNAs (Qi et al., 2006).

1.3 Chromatin dynamics during plant sexual reproduction

1.3.1 Chromatin dynamics during male gametogenesis

The structurally and functionally different cell types are marked by their dimorphic chromatin states (Figure 9). The generative and sperm cell entail highly condensed chromatin, while the chromatin in the vegetative cell is decondensed, with gradual decrease of linker histone H1 (McCormick, 1993; Tanaka et al., 1998). Removal of the repressive mark H3K9me2 in vegetative cell at bicelluar/tricellular stages indicates a permissive epigenetic landscape is established, which is supported by the enrichment of RNA PolII allowing for transcriptional activation, while the weakly distribution of H3K4me2 and eviction of H3K9ac, two transcriptionally permissive marks, underlying a bivalent chromatin landscape is adopted in vegetative cell (Houben et al., 2011). Additionally, the vegetative nucleus also undergoes centromeric heterochromatin decondensation, with dispersed 180-bp centromeric repeats (180CEN) signals and reduced H3K9me2 levels possibly by downregulation of DDM1 (DECREASE IN DNA METHYLATION 1), resulting in disassembly of centrometric heterochromatin (Soppe et al., 2002; Probst et al., 2003; Schoft et al., 2009). By contrast, RNA polII is almost absent in the generative cell and sperm, suggesting a typical repressive transcriptional landscape is established, this may be at least partly contributed by the enhanced levels of H3K9me2 in the generative cell and sperm. However, the increase of the permissive marks H3K4me2 and H3K9ac, as well as the sharp reduction of the transcriptionally repressive mark H3K27me3, indicates that the epigenetic landscape reflecting both repressive and permissive state, is established in the generative cell and sperm (Houben et al., 2011).

Male gametogenesis is also accompanied by changes of histone H3 variant repertoire, with the distinct patterns established between the sperm and the vegetative. In *Arabidopsis*, the pattern of H3 variants for somatic cells are evicted in both sperm and vegetative cell, instead a few H3 variants are expressed. In the vegetative cell, only canonical H3.3 variants including HTR5 and HTR8, as well as HTR14 are present, which is accompanied by erasure of CENH3

and disruption of centromeric heterochromatin, while the HTR5, the sperm-specific HTR10, and centromeric variant CENH3 are enriched in the sperm, reflecting the dimorphic patterns between the sperm and the vegetative cell. Both of these cell types are devoid of H3.1. The established H3 variants will not be inherited to the next generation as they will be reset and replaced by somatic patterns via de novo synthesis (Ingouff et al., 2007; Schoft et al., 2009). The dynamics of core histone variants is also described in Lily pollen, with the specific incorporation of gH2A, gH2B, as well as gH3 which shares common structural properties as Arabidopsis CENH3, in the generative cell, and enrichment of these histone variants in the sperm (Xu et al., 1999; Ueda et al., 2000).

Chromatin dynamics during male gametophyte development is also reflected by the distinct DNA methylation patterns established between the vegetative cell and the gametes, which can be traced back to the stage before mitosis I. The microspore lost CHH methylation mostly from the targets of retrotransposon loci. The sperm inherits DNA methylation patterns from the microspore, with further CHH demethylation, but retains CG and CHG methylation. The absence of DRM2 activity and genes required for 24nt siRNA biosynthesis in the microspore and the sperm, both of which are required for the maintenance of CHH methylation, may contribute to the sharp reduction of CHH methylation, while the low levels of DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1) and the homologs is likely to be important for the constant CG methylation (Law and Jacobsen, 2010; Calarco et al., 2012). DME and ROS1 are known as the DNA glycosylases that are responsible for erasure of methylated cytosine via a base excision repair process, thereby contributing to DNA demethylation (Morales-Ruiz et al., 2006). By contrast, the vegetative cell restores CHH methylation from TE loci via 24nt siRNA directed DNA methylation pathway, but lost CG methylation from the targets including DME, ROS1, DEMETER-LIKE2 (DML2) and DEMETER-LIKE3 (DML3), while the high expression level of DME may account for the CG demethylation in the vegetative cell.

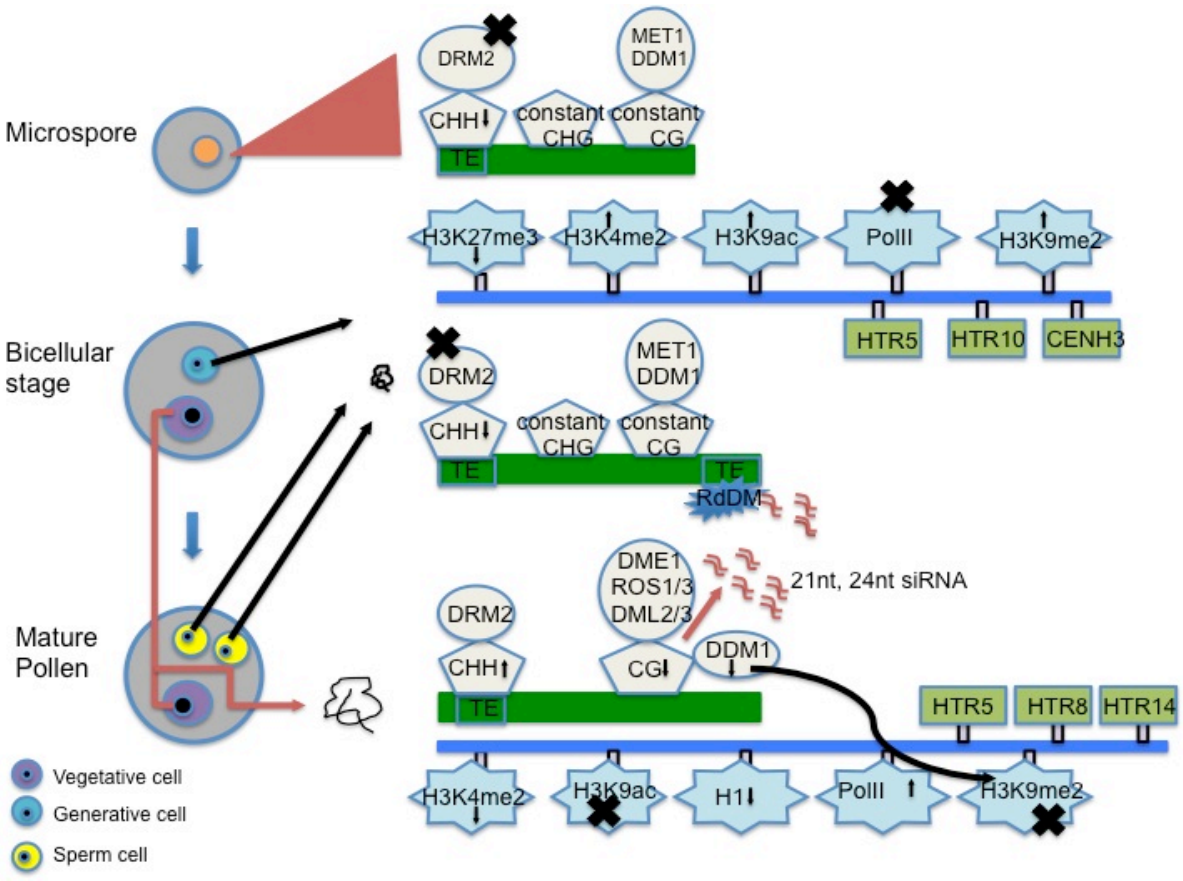

Figure 9. Dimorphic chromatin landscapes between the sperm nuclei and the vegetative nucleus. The sperm chromatin is condensed, with accumulating H3K9me2, H3K4me2 and H3K9ac, but absence of RNA polII indicating transcriptional quiescence. It undergoes dynamic changes of histone variants, with HTR5, HTR10 and CENH3 enriched in the sperm. Further, the sperm chromatin lost CHH methylation, but retains high level of CG and CHG methylation. By contrast, chromatin in the vegetative nucleus is less condensed, with eviction of linker H1, H3K9me2, H3K9ac, as well as CENH3, and weakly distribution of H3K4me2, but enrichment of PolII allowing for active transcription. It restores CHH methylation, but lost CG methylation at target loci mediated by DME, which in turn activates siRNA from TE in the vegetative cell. The siRNA could transpose to the sperm to reinforce TE silencing there.

1.3.2 Functions of chromatin dynamics during male gametogenesis

1.3.2.1 Epigenetic reprogramming in sperm cells

Unlike that in female gametes, the male gametes harbor identical genetic and chromatin state, without epigenetic differentiation between sperm cells. The repressive chromatin state of the male gametes, reflected by accumulating H3K9me2, absence of PolII, as well as constant CG

and CHG methylation, will be passed to the zygote after fertilization, thus the inherited transcriptional quiescent state from the male gamete might be important to reset the epigenome of the zygote to a ground sate, thereby enabling the zygote to acquire totipotency.

The high levels of CG methylation mediated by MET1 in the sperm cell possibly contribute to epigenetic inheritance. Methylated paternal alleles *FWA* and *FIS2* by MET1 in sperm cells will be passed to the endosperm following double fertilization, thereby promoting the paternal silencing of these imprinted genes. Thus, it plays pivotal roles in genomic imprinting in the endosperm (Saze et al., 2003; Jullien et al., 2006).

1.3.2.2 Mobile siRNA directed TE repression in sperm cells

Unlike that of the sperm cell, the vegetative cell doesn't contribute to its genetic information to the next generation. DME-dependent DNA demethylation leads to the reactivation of TE and generation of related siRNA in the vegetative nucleus, which is consistent with the downregulation of DDM1. It is of note that the accumulation of 21nt siRNA from Athila retrotransposons are also detected in the sperm, suggesting that siRNA generated upon TE reactivation in the vegetative cell may transpose to the sperm to reinforce TE silencing there, thereby contributing to genome integrity of the next generation (Schoft et al., 2009; Slotkin et al., 2009; Calarco et al., 2012; Ibarra et al., 2012).

1.3.3 Chromatin dynamics during female gametogenesis

During gametogenesis, the functional megaspore undergoes three rounds of mitosis to give rise to the female gametophyte harboring two gametes, the egg cell and the central cell, as well as five accessory cells.

Although both of these cells harbor less condensed chromatin compared to that of the somatic cells, dimorphic epigenetic landscapes are established between the egg cell and the central cell, which is similar to that in the male gametes (Figure 10). The chromatin is decondensed in the central cell, which is characterized by DNA demethylation due to DME, low levels of H3K9me2, as well as the increase of PolII recruitment following cellularization, allowing for active transcription (Pillot et al., 2010; Baroux et al., 2011). By contrast, the egg cell chromatin is condensed, with accumulated H3K9me2 and LHP1, as well as low levels of PolII immediately after fusion of the two polar nuclei, reflecting a quiescent state established in the egg (Pillot et al., 2010). The enrichment of H3K9me2 was caused by the recruitment of CMT3, resulting in silencing of TE in the egg (Pillot et al., 2010a; Pillot et al, 2010b).

The dimorphic epigenetic state between the egg cell and the central cell is also reflected by the distinct core histone variants patterns established. Like that in the male gametes, both of the female gametes are devoid of most of the H3 variants in the somatic cells. The mature egg cell only harbors HTR5, while the central cell retains one H3.1 variant (HTR3) and two H3.3 variants (HTR8 and HTR14). It is considered that the absence of H3.1 in the egg cell may be caused by the arrested cell cycle before S-phase, as H3.1 is tightly linked with DNA synthesis. The erasure of most H3 variants in both gametes may be important for limiting the inheritance of epimutations carried by different H3 variants (Ingouff et al., 2010). The specific eviction of core histone H2B in the egg cell, rather than in the central cell, further underlying the epigenetic dimorphism between the gametes (Pillot et al., 2010).

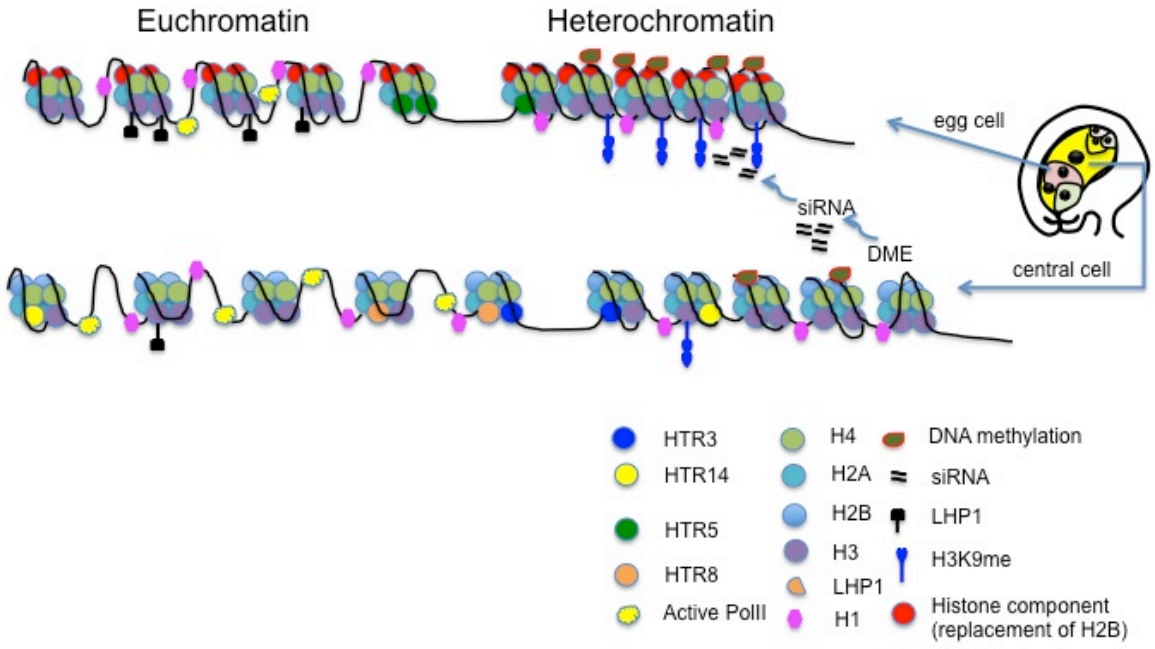

Figure 10. Dimorphic chromatin dynamics in the embryo sac. Chromatin of the egg cell is condensed, with accumulated LHP1, H3K9me2, and histone variant HTR5, but eviction of H2B and low enrichment of active (Ser2 phosphorylated) polII, suggesting a repressed transcriptional state. While, the central cell is decondensed, accompanied with loss of H3K9me2, but enriched polII allowing permissive transcriptional state. It undergoes DNA demethylation, with activation of TE related siRNA that could transpose to enhance TE repression in the egg.

1.3.4 Functions of chromatin dynamics during female gametogenesis

1.3.4.1 Further epigenetic reprogramming towards totipotency

The distinct chromatin state in the egg cell and the central cell implies that a dimorphic epigenetic landscape is established between these cell types. The quiescent state of the egg cell inherited by the zygote after fertilization will be critical for the acquisition of the totipotency enabling formation of all cell types by the zygote. Unlike that of the egg cell, the chromatin state of the central cell will not be transmitted to the next generation. By contrast, the permissive chromatin state in the central cell will be passed to the nursing endosperm during fertilization, while the transcripts will be provide for the embryo development, thereby indirectly contributing to the acquisition of totipotence for the embryo (Ibarra et al, 2012).

1.3.4.2 Erasure of epigenetic marks

Inheritance of DNA methylation across generations is important for repressing TE, thereby keeping genome integrity against aberrations. However, DNA methylation may induce transcriptional repression, thus confer the epigenetic barrier for development. How plants overcome this contradiction? DNA demethylation in the central cell leads to transcriptional activation, which can be transmitted to the endosperm, thereby promoting embryo development without running risk of passing the activated TE to the next generation, while the DNA in the egg cell is highly methylated.

DNA demethylation in the central cell is, at least in part, mediated by DME (Ibarra et al., 2012). In addition, the Retinoblastoma Pathway is also involved in passively DNA demethylation in the central cell. The human Retinoblastoma protein (pRb), binded by the partner RbAp48, is known to inhibit gene expression via repressing S-phase gene transcription (Nicolas et al., 2001). The interaction between the Arabidopsis homolog of pRb RETINOBLASTOMA RELATED 1 (RBR1) and of RbAp48 MULTICOPYSUPPRESSOR OF IRA1 (MSI1) in the MET1 promoter can repress MET1 transcription, thereby releasing the genes repressed by MET1 mediated DNA methylation (Jullien et al., 2008; Jullien and Berger, 2010). Thus, both passive and active mechanisms are employed to establish a permissive transcriptional state in the central cell, with activation of genes repressed by DNA methylation. The activation of maternally imprinted genes FWA and FIS2 in the central cell are dependent on the derepression of MET1 through the Retinoblastoma pathway and DME

activity, which will be inherited to the endosperm, thereby resulting in the maternal imprinting of these two genes (Choi et al., 2002; Kinoshita et al., 2004; Jullien et al., 2006). In addition, DME mediated DNA demethylation may be also required for activation of another maternal imprinted gene MEDEA (MEA) and the paternal imprinted gene PHERES1 (PHE1) (Xiao et al., 2003; Makarevich et al., 2008). The activated maternal MEA and FIS2 will facilitate the formation of FIS-PRC2 polycomb complex which recruits H3K27me3 to silence the maternal PHE1 allele, thereby contributing to the paternally imprinting of PHE1 (Kohler et al., 2003; Kohler et al., 2005). These evidences indicate that reprogramming of DNA methylation plays crucial role in general genomic imprinting in the endosperm.

1.3.5 Chromatin dynamics following double fertilization

1.3.5.1 Dimorphic chromatin landscapes established in two fertilization products

Plant sexual reproduction is marked by double fertilization, where the egg cell units with one sperm to produce the diploid zygote that will give rise to the embryo; the central cell fuses with the other sperm to give rise to the triploid endosperm. Thus, cell fates are transformed from the haploid gametes to the diploid zygote and triploid endosperm by uniting the parental genomes.

The zygote is transcriptionally quiescent, with barely detectable PolII activity and abundance of H3K9 dimethylation inherited from the egg; while the endosperm remains active transcription state, with enrichment of PolII, but removal of H3K9me2 even before the third division (Pillot et al., 2010). The endosperm chromatin is less condensed, with larger nuclei and nucleolus, as well as smaller sized chromocenters, which undergoes global DNA decondensation mediated by DME activity, accompanied with non-CG hypermethylation of the siRNA targeted sequence at local loci, while the zygote chromatin is relatively condensed, even though both entail decondensed chromatin compared to that of the somatic nuclei (Baroux et al., 2007b; Hsieh et al., 2009). The decondensed chromatin in the endosperm is marked by a peculiar heterochromatin organization, with the formation of additional heterochromatin foci (endosperm-specific interspersed ESI heterochromatin) dispersed into the euchromatin. Accompanying with this, the heterochromatin mark H3K9me1 is dispersed from the chromocenters to euchromatin and the interspersed heterochromatin, thereby more H3K9me1 is detected in enchromatin in most endosperm (Baroux et al., 2007b). Thus, the zygote/embryo and the endosperm entail dimorphic chromatin and transcriptional landscapes,

which is not surprising as the maternal genome is inherited from the epigenetically divergent egg and the central cell respectively.

Chromatin reprogramming in the two fertilization products are also reflected by the dynamic patterns of H3 variants established. The zygote erases gametic specific H3.3 variants possibly via a DNA replication independent pathway, with the incorporation of both parental genomes; while the depletion of inherited H3.3 variants in the endosperm is due to the dilution by DNA replication, with the segregation of paternal genome from the maternal genome. It indicates that the paternal genome may be reprogrammed in a distinct way compared to the maternal genome in the endosperm, which may be linked with genomic imprinting (Ingouff et al., 2007). Instead, the somatic patterns of H3.3 variants are reestablished in the zygote mediated by de novo synthesis of H3 variants. Reprogramming of H3.3 variants in the double fertilizations may contribute to the acquisition of totipotency of the zygote (Ingouff et al., 2010)

1.3.6 Functions of chromatin dynamics following double fertilization

1.3.6.1 Epigenetic reprogramming mediated by DNA methylation in the endosperm

DME directed DNA demethylation is important to ensure the active transcriptional state of the maternally imprinted genes like MEA, FWA, FIS2, as well as the expression of the paternally imprinted gene PHE1 in the endosperm (Choi et al., 2002; Xiao et al., 2003; Gehring et al., 2006; Jullien et al., 2006; Makarevich et al., 2008). This indicates that the extensive DNA demethylation may be generally important for the maintenance of maternally or paternally imprinted gene expression inherited from the mature gametophyte. The TE derived siRNA activated by extensive DNA demethylation possibly moves to the zygote to reinforce the silencing of TE, thereby ensuring the genome integrity across generations. The siRNA produced by the endosperm is also important to repress transcription in the zygote via siRNA directed CHH methylation, thus contributing to the acquisition of totipotency of the zygote and initiation of embryogenesis (Hsieh et al., 2009).

1.3.6.2 Epigenetic reprogramming during embryo development

During early embryogenesis of 2-4 cell stage, the transcriptome is predominantly contributed by the maternal gene expression, while the paternal transcription is repressed. The limited

paternal contribution at early stages is controlled by H3K9 dimethylation via RdDM pathway that represses the paternal transcription (Autran et al., 2011). The H3K9 dimethylation is mediated by the SUVH4 histone methyltransferase KRYPTONITE (KYP), while the siRNA for the RdDM pathway is maternally derived. The paternal gene contribution is increased at the globular stage. It is activated by the maternal histone chaperone complex CAF1 possibly via regulating the incorporation of the histone variants that are linked with transcription activity (Autran et al., 2011). The high levels of methyltransferases including MET1, DRM2 and CMT3 expressed in the embryo suggest that DNA methylation could be involved in reprogramming of the embryo via contributing to the quiescent transcriptional state in the zygote (Jullien et al., 2012). CHH methylation is increased in the heart and torpedo embryos, rather than CG and CHG methylation, via siRNA directed DNA methylation mediated by the methyltransferases DRM1 and DRM2. The siRNA may be transposed from the DNA demethylation of the endosperm or *de novo* synthesized by itself, or even from the male gametes. The enhanced CHH methylation in the embryo possibly plays important roles during development. It may repress the expression of certain imprinted genes which are activated in the early embryo, or contribute to genome integrity via silencing the transposon (Jullien et al., 2012). It is also likely to be involved in the acquisition of totipotency of the embryo to form all cell types via resetting certain epigenetic information.

1.4 Regulation of sporogenesis in plants.

Molecular and genetic analysis uncovered that sporogenesis during plant sexual reproduction was regulated by diverse factors. In *Arabidopsis*, *SPOROCYTLESS*/*NOZZLE* (*SPL*/*NZZ*) is critical for initiation of spore mother cell fate, with failure in acquisition of both male and female spore mother cell fate in *spl*/*nzz* mutant (Schiefthaler et al., 1999; Yang et al., 1999; Balasubramanian and Schneitz, 2000). The transcription factor NZZ is required for driving the expression of the shoot meristem stem cell regulator *WUSCHEL* (Koszegi et al.) which in turn indirectly activates the expression of the *WINDHOSE1* (*WIH1*) and *WIH2*, while *WIH* genes encode small uncharacterized peptides which promote female sporogenesis via interacting with the tetraspanin-type protein TORNADO2 (Lieber et al., 2011). While the *EXCESS MICROSPOROCYTES1 (EMS1)*, which encodes a putative leucine-rich repeat receptor protein kinase, is required for controlling somatic and reproductive cell fates in the *Arabidopsis* anther, mutants lacking EMS1 activity result in formation of excess microsporocytes and cytokinesis arrest that in turn causes defected microporogenesis and

male sterility (Zhao et al., 2002). Besides, the rice *MSP1*(*MULTIPLE SPOROCYTE*) (Nonomura, 2003) and maize *Multiple Archesporial Cells1* (*MAC1*) (Sheridan et al., 1996; Sheridan et al., 1999; Wang et al., 2012) play crucial roles in restricting the number of cells entering both male and female sporogenesis, with their mutation resulting in plural SMCs during early anther and ovule development. The rice *TAPETUM DETERMINANT1* like gene (*OsTDL1A*) was shown to interact with *MSP1* to restrict megaspore mother cell fate into a single cell in rice ovule primordium (Zhao et al., 2008).

The ARGONAUTE proteins are known as important players in the processes of microRNAs (miRNAs) and small-interfering RNAs (siRNAs) directed post-transcriptional gene silencing (PTGS) and RNA directed DNA methylation (Vaucheret, 2008). In Arabidosis, AGO9 is required to restrict germline fate into a single cell via a small RNA pathway (Olmedo-Monfil et al., 2010). Consistent with this, the maize *AGO104*, which belongs to the same AGO-clade as *Arabidopsis AGO9*, is essential for promoting meiosis during female sporogenesis, *AGO104* mutation causes unreduced embryo sac (Singh et al., 2011). While the rice AGO gene *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*), is essential for meiosis during sporogenesis, with an arrest at early Prophase 1 in the mutant lacking *MEL1* activity. It is noteworthy that some of the *mel1* male spore mother cell that arrested at leptotene or zygotene are characterized by reduced H3K9me2 intensity and altered nucleolar organizing region (NOR), suggesting chromatin reprogramming may play important roles during sporogeneis (Nonomura et al., 2007). While *Arabidopsis AGO5* is also required to promote female gametophyte via small RNA pathway (Tucker et al., 2012). These studies suggest that chromatin remodeling through ARGONAUTE mediated small RNA pathway is likely to play essential roles during sporogenesis. In addition, transcriptome profiling of the female spore mother cell uncovered that a novel RNA helicase, MEM, is required for sporogenesis, gametogenesis and embryogenesis, with additional enlarged female spore mother cells formed per ovule primordium in *mem* mutant phenocopying *AGO9* mutant. It is of note that 40% and 33% of arrested ovules and aborted seeds were observed in *mem-1* and *mem-2* mutants, with higher order chromatin structure and LIKE HETEROCHROMATIN PROTEIN1 (LHP1) distribution affected in *mem* gametophytic nuclei suggesting chromatin remodeling are involved in key steps of plant sexual reproduction (Schmidt et al., 2011).

2. Aims of the Thesis

Plant sexual reproduction is marked by several cell fate transitions: during sporogenesis, gametogenesis, and embryogenesis. Genetic analyses uncovered several molecular factors regulating cell identity establishment where epigenetic mechanisms emerged as fundamental players during sporogenic fate acquisition and post-meiotically during gametophyte development and seed development (Introduction). Early observations, eg in the 1950's, that SMC differentiation was accompanied by visible changes in nuclear phenotype suggested, in the light of our current understanding, large-scale chromatin reorganization. My PhD project was motivated by the working hypothesis that the somatic-to-reproductive cell fate transition may be underlined by chromatin reprogramming. Previous work that had been conducted in C.Baroux' group suggests specific histone dynamics in the MMC, thus prompting us to characterize chromatin organization in details during MMC differentiation. However, research investigations were largely hindered due to the relative inaccessibility of female MMC enclosed in the ovule, located inside the carpel. Thus, the aims of my PhD were to:

- Develop an efficient method for quantitatively analyzing chromatin organization at high-resolution, in single MMC in whole-mount plant ovules (Chapter I).

- Elucidate whether chromatin reprogramming underlies the somatic-to-reproduction cell fate transition during the differentiation of MMC in *Arabidopsis* (Chapter II).

- Develop an approach to profile the MMC epigenome and identify the genomic loci targeted by chromatin reprogramming (Chapter III).

- Determine whether chromatin reprogramming also underlies PMC differentiation in *Arabidopsis* (Chapter IV).

- Determine whether chromatin reprogramming during MMC development is an evolutionary conserved scenario among monocot plants, taking rice (*Oryza sativa*), as a model system (Chapter V).

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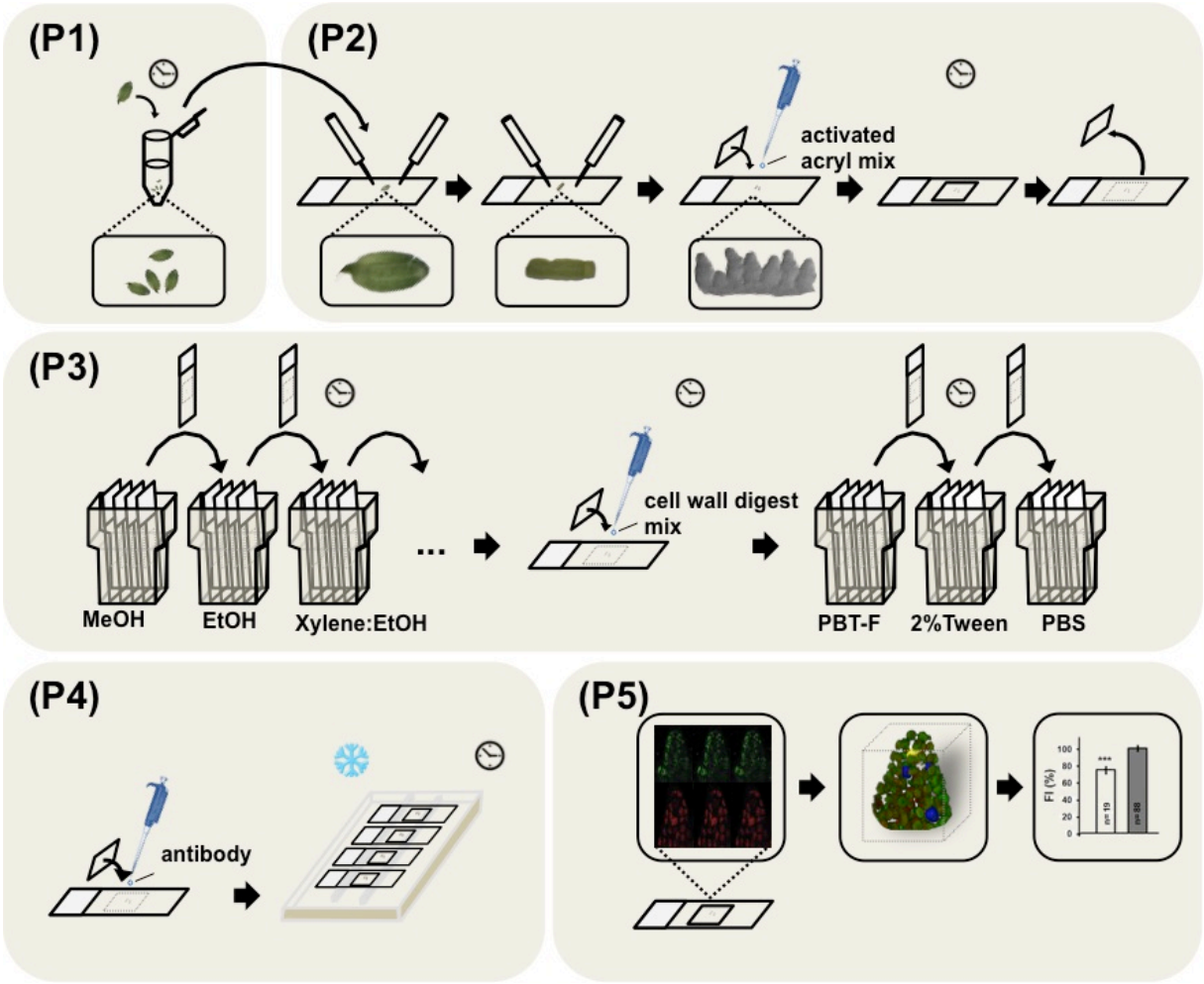
3. Result Chapter I

Robust and Efficient method for quantitative Single-Cell Analysis of Chromatin Modification and Nuclear Architecture in Whole-Mount *Arabidopsis* Ovules

Sexual reproduction in flowering plants is initiated by the establishment of the reproductive lineage in specialized reproductive structures (the female ovule and the male sporangium). The male reproductive lineage remains a relatively accessible object-of-study, while limited accessibility of female reproductive cells and their precursors, which are deeply embedded inside the ovule, itself enclosed in the flower carpel, technically impaired cytological and cytogenetic analyses.

Thus, we developed an efficient and robust method to analyze the nuclear organization and chromatin modification in the female reproductive lineage, at the single-cell level with high resolution, in whole-mount embedded Arabidopsis ovules. Our manuscript reporting this elegant method was accepted by *Journal of Visualized Experiments*, with peer-review. It is now *in Press*. A video, showing the method, will be produced in March, 2014, and the figure describing the workflow is shown in the next page (depicted by C. Baroux). I contributed to the results involved in Figure 1, 2, 3, 4, 5A and Figure 6 as part of my PhD work, and wrote the manuscript, together with C. Baroux. It is here presented in its accepted format.



(P1) Fix fresh flower buds in a fixative solution.

(P2) Dissect and embed ovules in miniature acrylamide pads directly on microscopy slides.

(P3) A series of tissue processing enabling tissue clarification and permeabilization.

(P4) Incubate the treated samples with an antibody solution for immunostaining or a labeled probe for fluorescent in situ hybridization.

(P5) Confocal imaging at high-resolution followed by 3-dimensional reconstruction allows for quantitative analyses in whole-mount at the single cell level.

An efficient method for quantitative, single-cell analysis of chromatin modification and nuclear architecture in whole-mount ovules in *Arabidopsis*

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Keywords:
Arabidopsis thaliana, ovule, chromatin modification, nuclear architecture, immunostaining, Fluorescence In-Situ Hybridization, DNA staining, Heterochromatin

Short Abstract:

We provide here an efficient and reliable protocol for immunostaining, Fluorescence In-Situ Hybridization, DNA staining followed by quantitative, high-resolution imaging in whole-mount *Arabidopsis thaliana* ovules. This method was successfully used to analyze chromatin modifications and nuclear architecture.

Long Abstract:

In flowering plants, the somatic-to-reproductive cell fate transition is marked by the specification of spore mother cells (SMCs) in floral organs of the adult plant. The female SMC (megaspore mother cell, MMC) differentiates in the ovule primordium and undergoes meiosis. The selected haploid megaspore then undergoes mitosis to form the multicellular female gametophyte, which will give rise to the gametes, the egg cell and central cell, together with accessory cells. The limited accessibility of the MMC, meiocyte and female gametophyte inside the ovule is technically challenging for cytological and cytogenetic analyses at single cell level. Particularly, direct or indirect immunodetection of cellular or nuclear epitopes is impaired by poor penetration of the reagents inside the plant cell and single-cell imaging is demised by the lack of optical clarity in whole-mount tissues.

Thus, we developed an efficient method to analyze the nuclear organization and chromatin modification at high resolution of single cell in whole-mount embedded Arabidopsis ovules. It is based on dissection and embedding of fixed ovules in a thin layer of acrylamide gel on a microscopic slide. The embedded ovules are subjected to chemical and enzymatic treatments aiming at improving tissue clarity and permeability to the immunostaining reagents. Those treatments preserve cellular and chromatin organization, DNA and protein epitopes. The samples can be used for different downstream cytological analyses, including chromatin immunostaining, Fluorescence In-Situ Hybridization (FISH) and DNA staining for heterochromatin analysis. Confocal laser scanning microscopy (CLSM) imaging, with high

resolution, followed by 3D reconstruction allows for quantitative measurements at single-cell resolution.

Introduction:

In flowering plants, the establishment of reproductive lineages begins with the differentiation of SMCs, female MMC and male microspore mother cell. The MMC develops from a sub-epidermal nucellar cell at the distal tip of the ovule primordium, and the microspore mother cell develops from sporogenous tissue in the anther locule, which are located deep inside the floral organs[1]. SMCs undergo meiosis to produce haploid spores, which then give rise to the gametophytes upon mitosis. The female gametophyte, or embryo sac, consists of one egg cell, one central cell, two synergids and three antipodals. The male gametophyte, or pollen, is composed of one vegetative cell and two sperm cells. While the male gametophyte remains a relatively accessible object-of-study, the female gametophyte is embedded inside the ovule, itself enclosed in the flower carpel, and thus poses specific challenges to molecular and cytological analyses. Recently, however, laser-assisted microdissection offered an elegant solution allowing transcriptomic analyses in the MMC and female gametophytic cells[2-4]. In addition to candidate gene expression analyses, using e.g. RNA *in situ* hybridization or reporter gene assays, cytological analyses allows investigating the dynamics of endogenous cellular components using specific direct cellular staining or indirect immunostaining. Particularly, cytogenetic staining using FISH and DNA staining, together with immunostaining of chromatin modifications or chromatin components are central approaches to elucidate chromatin dynamics and nuclear organization in Arabidopsis[5]. Typically, meiosis entails specific chromosome dynamics which has been well investigated in plant male meiocytes[6,7]; further large-scale, cell-specific chromatin reorganization, likely reflecting dynamic epigenetic reprogramming has been described during pollen development[8-10]. By contrast, due to the relative inaccessibility of the female meiocyte and gametophyte, these investigations remain technically difficult to apply, and often require sectioning or manual dissection and enzymatic digestion (see below). In addition, the prevalent lack of optical clarity in whole-mount is an obstacle to high-resolution imaging of reproductive cells in intact ovules.

A classical method for cytological analysis of chromosome organization in whole-mount ovules uses Feulgen's staining[11-13]. It involves acid hydrolysis (using hypochloric acid) of the DNA which results in protein denaturation and thus causes destruction of the chromatin structure. Alternatively, chromosome organization in female meiocytes and gametophytic cells can be observed using DAPI staining and immunostaining on semi-thin sections or dissected embryo sacs and MMC (for instance see[14-18]). Clearly, however, manual dissection and sectioning can be labor intensive and impedes on the qualitative and quantitative analysis of a large number of chromatin epitopes.

Here we provide an efficient protocol to prepare a large number of Arabidopsis ovules suitable for a variety of downstream cytological staining in whole-mount. In brief, flower buds are incubated in a fixative solution, rows of ovules are dissected from the carpel and embedded in acrylamide on slide as done for pollen meiocytes[19,20]. The embedded ovules are

further cleared and fixed in methanol, ethanol and xylene before cell wall digestion and permeabilization. Possible variations of these steps are discussed. The samples can then be used for DNA staining, immunostaining and FISH. The preparation mode is efficient and allows for parallel experimental set-up (up to 16 slides can be prepared in a day for different downstream analysis). The treatments described enable homogeneous signals in whole-mount and well-preserved histological, cellular and nuclear organization in reproductive cells and surrounding nucellar cells which benefit qualitative and quantitative comparisons between cell types. Calibrated, CLSM-based high-resolution imaging followed by 3-dimensional reconstruction enables meaningful quantitative measurements of fluorescent signals. We successfully used this procedure to analyze chromatin dynamics in the differentiating MMC[21] and developing female gametophyte[22]; we present here representative results of heterochromatin analysis, chromatin immunostaining, GFP immunostaining and FISH in whole-mount ovules. We further believe that our protocol will be suitable for other plant tissues and species.

Protocol:

Note: The procedure is described in the workflow in Figure 1, and the setup for dissection and embedding of tissues are presented in Figure 2.

1. Tissue Fixation

1.1 Collect 20-30 carpels in a microfuge tube containing freshly made BVO fixative buffer on ice.

1.2 Fix the tissue 30 min with gentle shaking at room temperature.

1.3 Spin the tubes containing the carpels in fixative in a benchtop microcentrifuge 1 min at 400 × g.

1.4 Remove carefully the fixative buffer and add 1 mL of PBT, place the tubes on ice.

2. Dissection and embedding

2.1 Prepare five eppendorf tubes with each 200 μL of a freshly made, 5% acrylamide mix.

2.2 Prepare five Superfrost slides pre-cleaned with 70% ethanol and labeled with a pencil.

2.3 Thaw one aliquot of 20% APS and 20% NaPS each, on ice.

2.4 Take 4-5 carpels with a cut-end tip, place them on a clean slide, remove the excess of liquid.

2.5 Make longitudinal cuts with a fine needle and detach the carpel walls to release rows of ovules as shown Figure 2, avoid drying by covering with PBS (not more than 10 µL).

2.6 Quickly add and mix 12 µL NaPS, 12 µL APS with an aliquot of 200 µL acrylamide mix.

2.7 Add 30 µL of the activated acrylamide onto the dissected ovules.

2.8 Cover with a 20 mm × 20 mm coverslip, let polymerize at room temperature, 45-60 min.

2.9 Remove the coverslip using a razor blade. At this stage, the samples can be kept overnight at 4 °C in a coplin jar containing PBS.

3. Tissue Processing

Note: All steps except 3.2.1, 3.2.3, 3.3.2 and 3.4.3 are carried out in coplin jars with 80 mL solution under the chemical hood at room temperature. Slides are transferred with a flat-tip forceps.

3.1 Tissue clarification and fixation

3.1.1 Incubate 5 min in methanol

3.1.2 Incubate 5 min in ethanol,

3.1.3 Incubate 30 min in ethanol:xylene (1:1)

3.1.4 Incubate 5 min in ethanol.

3.1.5 Incubate 5 min in methanol.

3.1.6 Incubate 15 min in methanol and PBT (1:1), complemented with 2.5% Formaldehyde.

3.1.7 Rinse 2 ×10 min in PBT. At this stage, slides can be kept overnight at 4 °C.

3.2 Cell wall digestion

3.2.1 Thaw an aliquot of the cell wall digestion mix on ice.

3.2.2 Take a slide from the coplin jar, drain the excess of liquid by placing it vertically on a paper towel.

3.2.3 Add 100 µL of cell wall digestion mix over the acrylamide pad and cover with a 23 mm × 46 mm coverslip. Repeat for the other slides. Incubate for 2 hrs at 37 °C in a moist chamber (described in Materials).

3.2.4 Wash the slides 2 × 5 min in PBT.

3.3 RNase A treatment

3.3.1 Take a slide from the coplin jar, drain the excess of liquid as before.

3.3.2 Incubate each slide with 100 μL of RNAseA at 100 μg/mL in PBS with 1% Tween-20 for 1 hr at 37 °C in a moist chamber.

3.3.3 Wash the slides for 2 × 5 min in PBT.

3.4 Post-fixation and permeabilization

3.4.1 Post-fix for 20 min in freshly made PBT-F.

3.4.2 Rinse the slides for 10 min in PBT.

3.4.3 Permeabilize for 2 hrs in PBS with 2% Tween-20 at 4 °C.

3.4.4 Rinse the slides for 2 × 5 min in PBT.

4. Immunostaining

Note: For this step, the optimal concentration of the primary antibody has to be tested by using different dilutions (1:200, 1:500, 1:1000) of the antibodies.

4.1 Incubate each slide with 100 μL of primary antibody diluted in PBS with 0.2% Tween-20 for 12-24 hrs at 4 °C.

4.2 Wash the slides in PBT for 2-4 hrs at room temperature under gentle shaking.

4.3 Apply the secondary antibody 1:200 in PBS + 0.2% Tween-20 for 24 hrs at 4 °C.

4.4 Wash slides in PBT for 1 hr at room temperature under gentle shaking.

4.5 Counterstain with 10 μg/mL propidium iodide in PBS for 15 min, then rinse 15 min in PBS under gentle shaking, at room temperature.

4.6 Mount in anti-fading liquid mountant supplemented with 10 μg/mL propidium iodide. Let the mounting medium harden for 1 hr before acquiring images by CLSM.

5. Quantitative Imaging

5.1. Image acquisition

5.1.1 Acquire high-resolution images using CLSM, ideally using a resonance scanning mode, which allows better preservation of fluorescent signals over prolonged imaging[23], and a 63× Glycerol immersion lens.

5.1.2 Test the acquisition parameters such as laser intensity, gain, pinhole, voxel size and zoom factor at the beginning of the experiment to define a standard acquisition procedure to strictly follow throughout all slides for consistent quantitative measurements.

5.1.3 Verify the absence of cross-talk between fluorochromes. If present, set up a sequential scan. Acquire transmission images separately and not simultaneously.

5.1.4 Perform serial, three-dimensional image acquisition with highest possible resolution in the x and y dimensions and with 2-times oversampling in the z dimension (Nyquist's rule).

5.2. Image processing

5.2.1. Reconstruct serial images in three-dimensions using commercial or open source software.

5.2.2. Define contour surfaces around each nucleus (or cell) of interest in 3D.

5.2.3 Quantify fluorescence in each channel as the sum of pixel intensities in each object.

5.2.4. Export the data to Excel for statistical analyses. Normalize antibody signals against e.g. DNA staining signals.

Representative Results

We provide a robust protocol for large-scale preparation and processing of Arabidopsis ovules suitable for cytological staining in whole-mount. Thanks to the embedding, the ovules retain a 3-dimensional structure (Figure 3). Furthermore, the tissue processing including optical clarification enables imaging subcellular structures at high-resolution. Figure 4 shows DNA staining in whole-mount ovule primordia where heterochromatin appears as bright, well defined conspicuous foci (no deconvolution was used for this picture). These images were used for analyzing heterochromatin content in the MMC and nucellus (Figure 4, [21]).

In addition, we successfully used this protocol to quantitatively analyze chromatin dynamics by immunostaining in megaspore mother cells, functional megaspore, developing female gametophytes and early embryo[21, 22-24]. In figure 5, we show representative results of whole-mount immunostaining on Arabidopsis ovules. Figure 5A shows an example of

immunodetection in ovule primordia, including the megaspore mother cell, of a euchromatin-associated permissive mark (H3K4me3) and a heterochromatin-associated repressive mark (H3K27me1). Figure 5B shows an example of GFP immunodetection in a mature ovule, including the embryo sac (in this case, the protocol was slightly modified for using the GFP booster antibody (see discussion). We also detected native chromatin proteins such as H3 and H1[21] showing that the procedure preserves chromatin protein epitopes. The procedure also allows for reproducible quantifications enabling comparison between cell types (e.g. reproductive vs somatic, surrounding cells, [21]).

Finally, we also successfully applied this procedure to carry-out FISH analyses on whole-mount ovule primordia. An example is shown Figure 6 showing FISH signals using a probe against 45S rDNA repeats defining the nucleolar organizing regions[25]. The DNA probe was directly labeled with Alexa 488 using FISH-Tag[26], hybridization was done essentially as described[27] with minor modifications, while DNA counterstaining was done as described in our protocol.

Figure Legends:

Figure 1. Workflow of immunostaining, DNA staining and Fluorescence In-Situ Hybridization in Arabidopsis ovules.

Figure 2 Setup for dissection and embedding of carpels on slide. The capel wall is removed and the carple is dissected on the slide to release rows of ovules (see close up of dissected ovules in step 3), and then the dissected carpel is embedded in activated acrylamide mix, covered by 20 mm × 20 mm coverslip.

Figure 3. The protocol enables preserving the 3-dimensional structure while allowing optical clarity and homogenous staining. The images shows a split section view of the 3D image in xy, xz and yz axis as indicated. The image data have been acquired by confocal laser scanning microscopy and reconstructed in 3 dimensions using the Imaris software.

Figure 4 Whole-mount DNA staining by propidium iodide in ovule primodia allows for precise heterochromatin quantification. The image on the left shows whole-mount DNA staining throughout an ovule primordia. An MMC nucleus is marked by a white contour and a nucellar nucleus in red. Projections of 3D-reconstructed nuclei are shown on the right. The clarity of the tissue enables high-resolution imaging of the heterochromatin foci marked by a yellow contour and quantification of the fluorescent signals therein. The graphs show the relative heterochromatin fraction[21].

Figure 5. Representative results of whole-mount immunostaining in Arabidopsis ovules.
A. Immunostaining of chromatin modifications in young ovule primordia detecting euchromatin (H3K4me3) and heterochromatin (H3K27me1). The antibody signal is green, the DNA counterstained by propidium iodide in red. An overlay of fluorescent signals is shown together with a picture in transmission light using differential interference contrast (grey). MMCs are indicated by white contours. A close-up of the MMC nucleus is shown as inset in

the upper panel. The images are single confocal section. B. Immunodetection of GFP in a mature ovule. The GFP was immunostained using GFP-booster antibody and the ovule was counterstained with DAPI.

Figure 6. Whole-mount Fluorescence In-Situ Hybridization in Arabidopsis ovules.
The ovule primordium was hybridized with a DNA probe specific to 45S rDNA repeat loci and labeled with Alexa488 using the FISH-Tag technology, and counterstained with DAPI[26]. The overlay of 45S rDNA with DAPI and image acquired in the transmission light channel (grey) are also shown. A close-up of the MMC nucleus is shown as inset.

Figure 7. Influence of the fixation, digestion and staining procedure on DNA signals in whole-mount ovules.
A. mature ovules were fixed 30 min either with 4% paraformaldehyde or BVO fixative and processed 30 min or 1 hr with cell wall digest enzyme mix before DNA staining with propidium iodide. For a given batch, longer incubation affects more negatively on the DNA staining ovules that were fixed with paraformaldehyde than with BVO. B. whole-mount DNA staining using the Feulgen reagent following a required acid-hydrolysis[28] or using propidium iodide following the non-denaturing protocol described in the text. The upper panel shows a single plane section through the embryo sac, the lower panel presents a magnification over the central cell nucleus showing clearly alteration of chromatin organization in Feulgen-stained ovules. ccn, central cell nucleus, ecn, egg cell nucleus, syn, synergid nucleus.

Discussion:

In flowering plants, the female reproductive lineage is surrounded by several cell layers including the nucellus and the ovule teguments, thus rendering cytological staining in whole-mount technically challenging. Here we present an efficient protocol enabling the preparation and processing of a large number of ovules suitable for cytological staining such as immunostaining, DNA staining and Fluorescence In-Situ Hybridization in whole-mount. We successfully used it for the analysis of the female reproductive germline in Arabidopsis[21,22]. This method is highly efficient as several slides can be treated in parallel for different staining. It is also robust and gives homogeneous signal distribution and allows for reproducible quantitative analyses. By contrast to classical method such as Feulgen staining which involves the denaturation of the chromatin structure, our protocol preserves chromatin organization and nuclear epitopes. In addition, tissue clarification enables imaging the signals at high resolution at the single-cell level.

This protocol can be expedited by omitting the steps described in 3.1 if the flowers have been fixed in 4% paraformaldehyde (in PBS + 1% Tween) instead of BVO buffer (1.1). While this shorter procedure proved functional for several immunostaining[22-24], we found that it attenuates the robustness of the staining across samples, antibody and batch of cell wall digestion enzyme mix (see below). Furthermore we were not successful for FISH hybridization with this short procedure. For immunodetection of GFP's using the booster molecules as shown Figure 5B, a similar protocol as described here was used with slight modifications at step 1 and 3: carpels were fixed in 2.5% Formaldehyde for 45 min (1.1), the

first step of tissue processing was shortened to 5-10 min methanol treatment (3.1), a blocking step was introduced (30 min in 2% BSA in PBS) prior to antibody application overnight as described. No secondary antibody is necessary with the booster.

We discuss below some critical steps:

Tissue Fixation, dissection and embedding.
Immunostaining and DNA staining signals were consistently more robust and homogenously distributed when the tissue was sampled from plants less than 5 weeks old (following transfer of the seedling in soil). Possibly, in our growth conditions, a prolonged period of cultivation may be accompanied by changes in the biochemical composition of the cell wall, influencing in turn the efficiency of tissue processing. Thus we recommend sampling tissue from relatively young plants. In addition, the tissue should be prevented from drying during dissection (leading otherwise to histological alteration and absence of staining signals) while an excess of PBS challenges the manipulation; a gentle draining of the excess of solution with the tip around the tissue deposited on slide is thus recommended. Furthermore, bubbles should be avoided in the acrylamide mixture and while covering with a coverslip. Finally, Superfrost Plus slides are strongly recommended for adequate acrylamide adhesion (standard quality lead to fragile and unstable pads in our hands), as they appear superior to others for tissue and acrylamide adhesion.

Fixation, permeabilisation and cell wall digestion.
Cell wall digestion is a critical step of tissue processing. It is thought that this step facilitates a good penetration of the staining reagents homogenously throughout the plant tissue. We experienced variability in staining homogeneity (ranging from no signal, signal in only part of the tissue, to 100% tissue staining) depending on the digestion time and the enzymatic activity (batch-specific, described by the provider). It is recommended to produce a large amount of stock solution of the enzyme mix (e.g. 100 mL) and keep 1 mL aliquots at -20 °C. Each stock solution should first be tested on 1-2 slides before using at large scale. Furthermore, we experienced that the type of fixative influences the efficiency of DNA staining in combination with different processing time for cell wall digest: ovules fixed in 4% paraformaldehyde were negatively affected by prolonged incubation with the cell wall digestion mix, while tissue fixed with the BVO solution were tolerant to longer digestion times and allowed better DNA staining (Figure 7A). In addition, an RNAse (DNase free) treatment is strictly necessary if the tissue is counterstained with propidium iodide as it also binds to RNA molecules. We advise against using 4',6-diamidino-2-phenylindole (DAPI) due to its broad fluorescent emission spectra overlapping with other fluorophores but also to achromatic aberrations[29] that necessitate channel shift corrections post-acquisition. We also recommend against Feulgen staining which requires an acid-hydrolysis during tissue processing[28] leading to chromatin denaturation particularly in the embryo sac (Figure 7B). Alternative DNA dyes may also be used[30], but their efficiency has not been tested here.

Immunostaining.
For immunostaining of chromatin modifications, we recommend to verify the specificity of the primary antibody in the open source database

http://compbio.med.harvard.edu/antibodies/projects/1,[31] as some commercially available antibodies showed cross-reactions for other modifications. For downstream quantification analyses, it is important to calibrate the antibodies concentration and incubation time to measure signals in a linear relationship with the epitope. We recommend to calibrate the antibody dilution and detection time using an antibody dilution series of 1:200, 1:500, 1:1000 and from 12 to 24 hrs incubation, respectively, to identify the conditions giving robust and homogenous signals. The highest dilution and shortest incubation time allowing reproducible signals should be used for quantitative analyses. Controls without primary antibody should be performed to test for the specificity. If immunostaining produces unspecific staining, it is advised to block with 5% BSA + 0.1% Tween in PBS for 2 hrs at 4 °C before applying the primary antibody. Antibody signals can be checked on the slide before DNA counterstaining to verify the success of the experiment. In our hands, washing in PBT for 2-4 hrs after incubation with the primary antibody, and 1 hr after the secondary antibody allows for low background signals even without blocking.

Finally, this protocol also is likely applicable to other plant tissues (e.g. root, leaf fragment, floral meristem) and probably to other plant species, providing some adjustment on the dissection, cell wall digest and permeabilization.

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Disclosures:

The authors declare that they have no competing financial interests.

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Figures

Figure 1

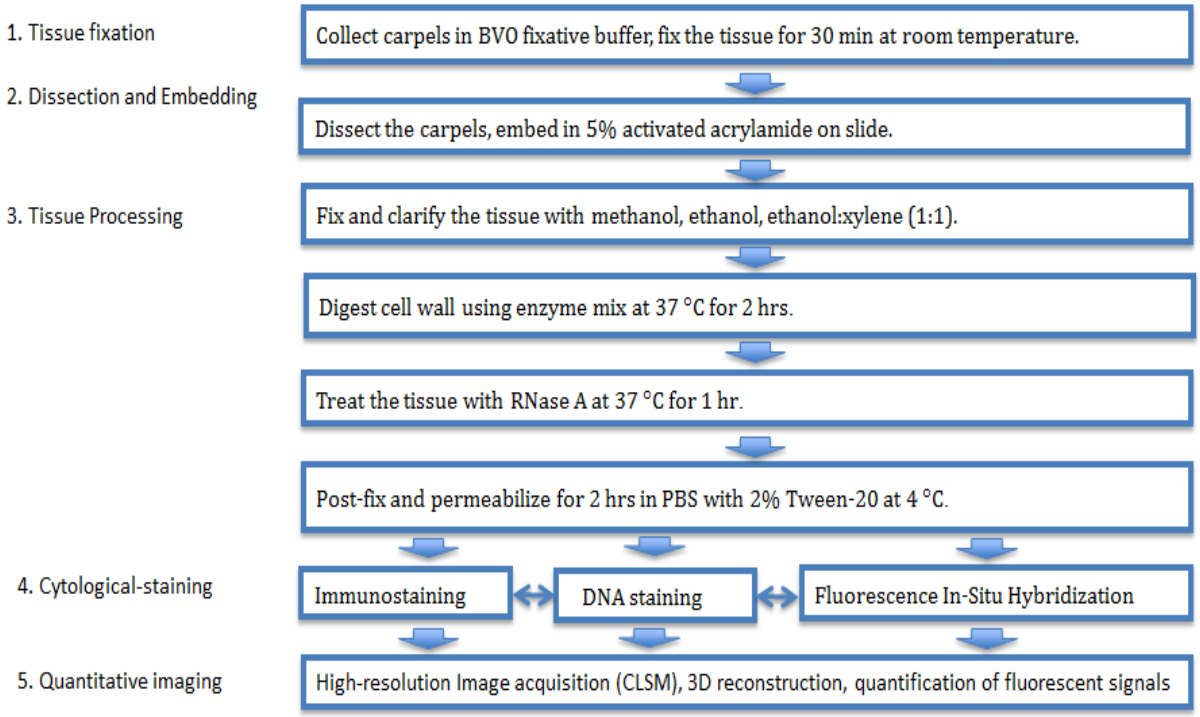


Figure 2

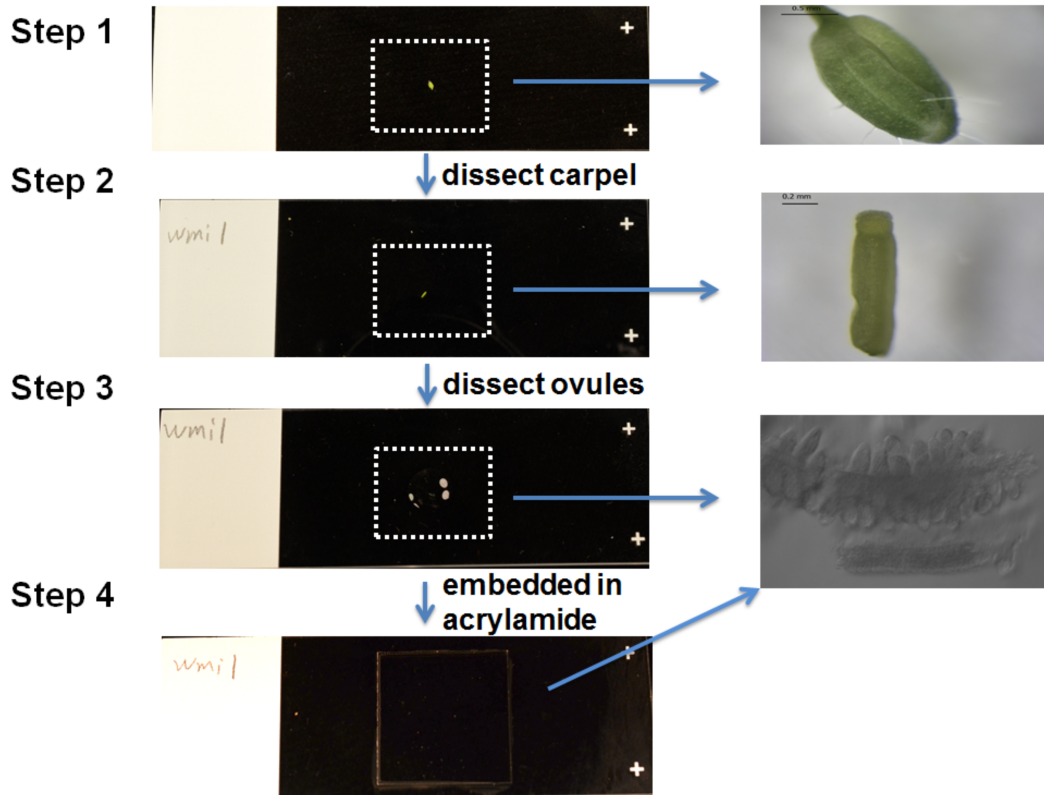


Figure 3

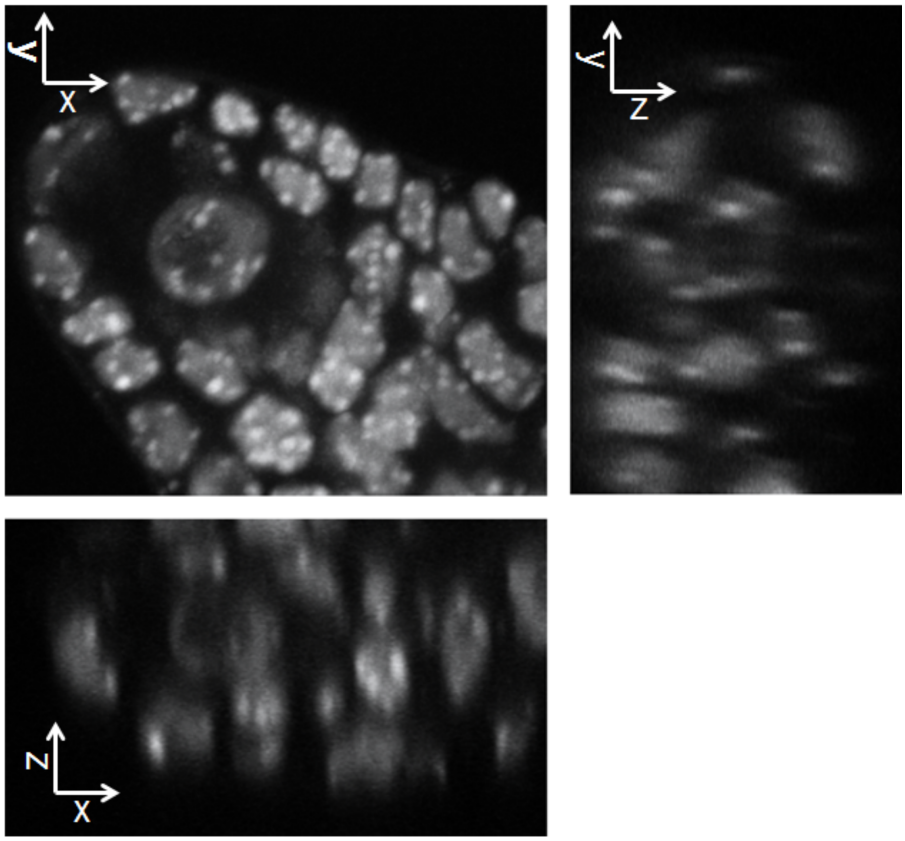


Figure 4

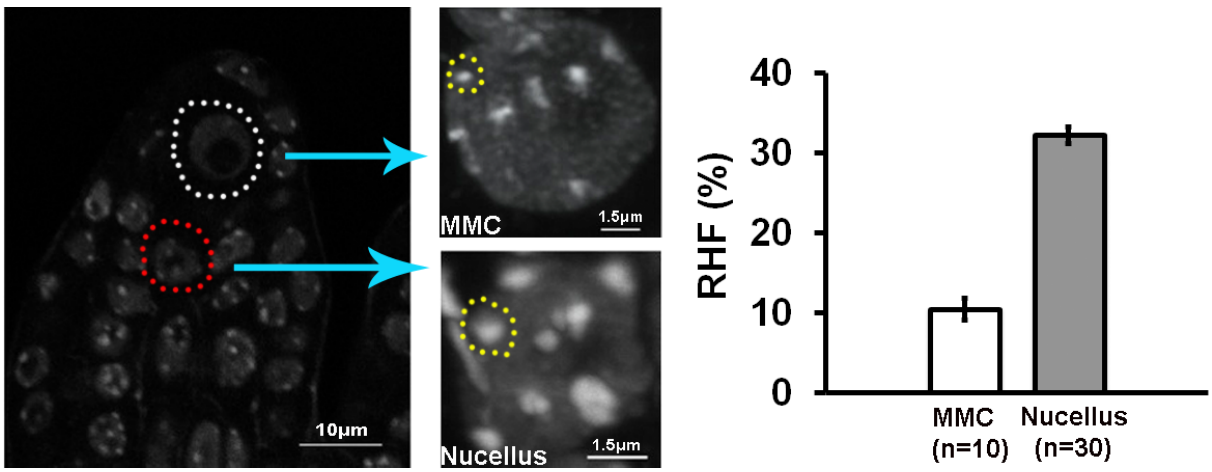


Figure 5

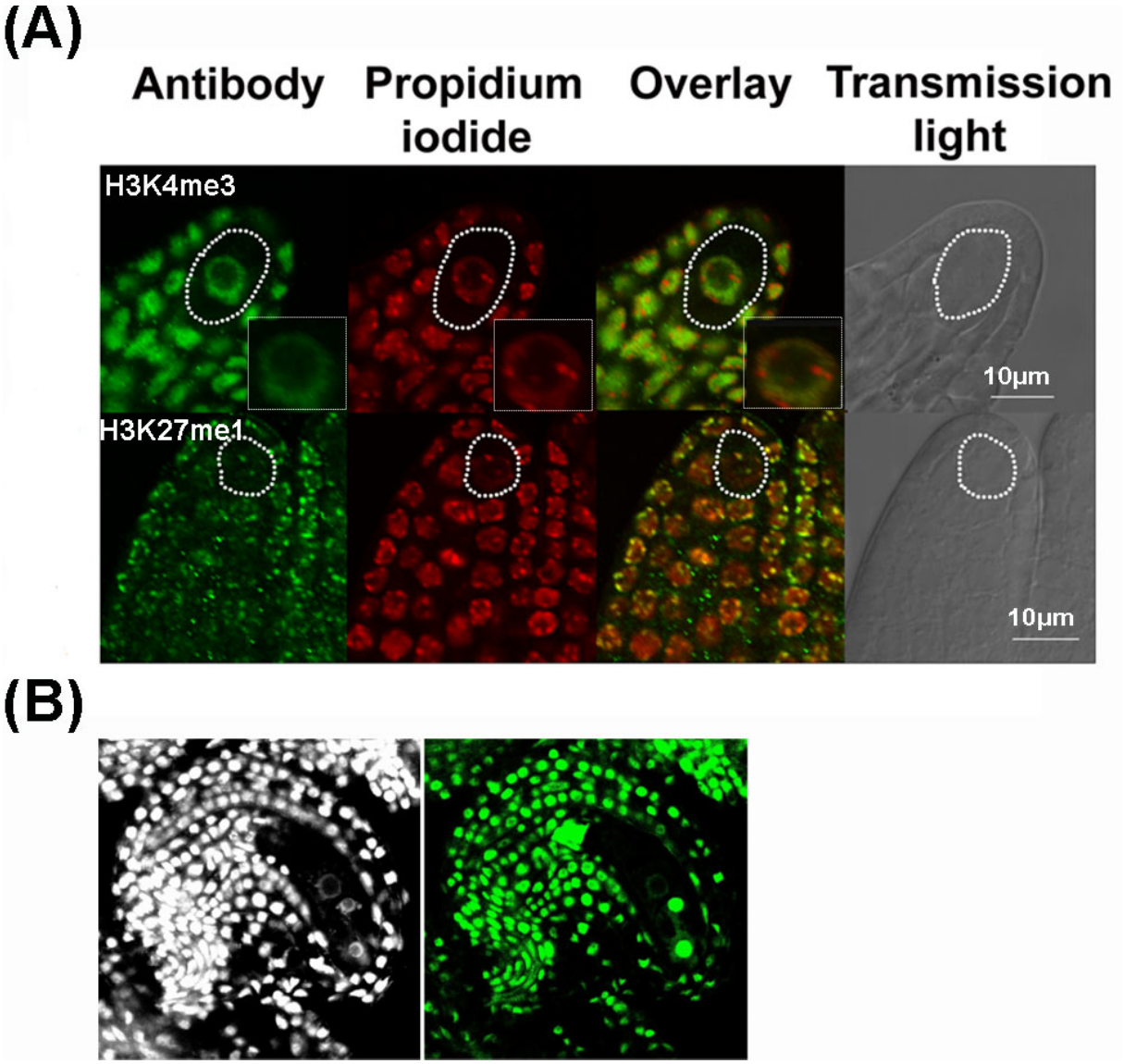

(A)
Antibody
Propidium iodide
Overlay
Transmission light
H3K4me3
H3K27me1
10µm
10µm
(B)

Figure 6

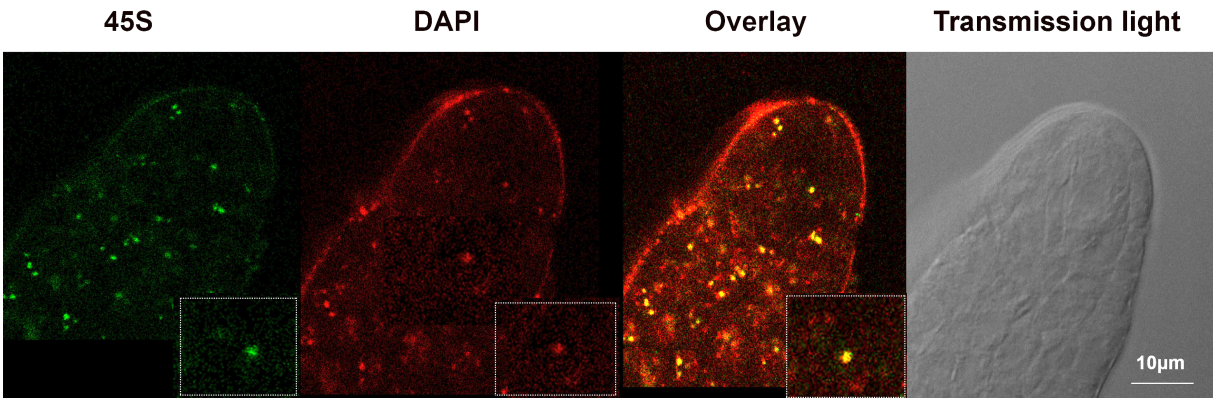
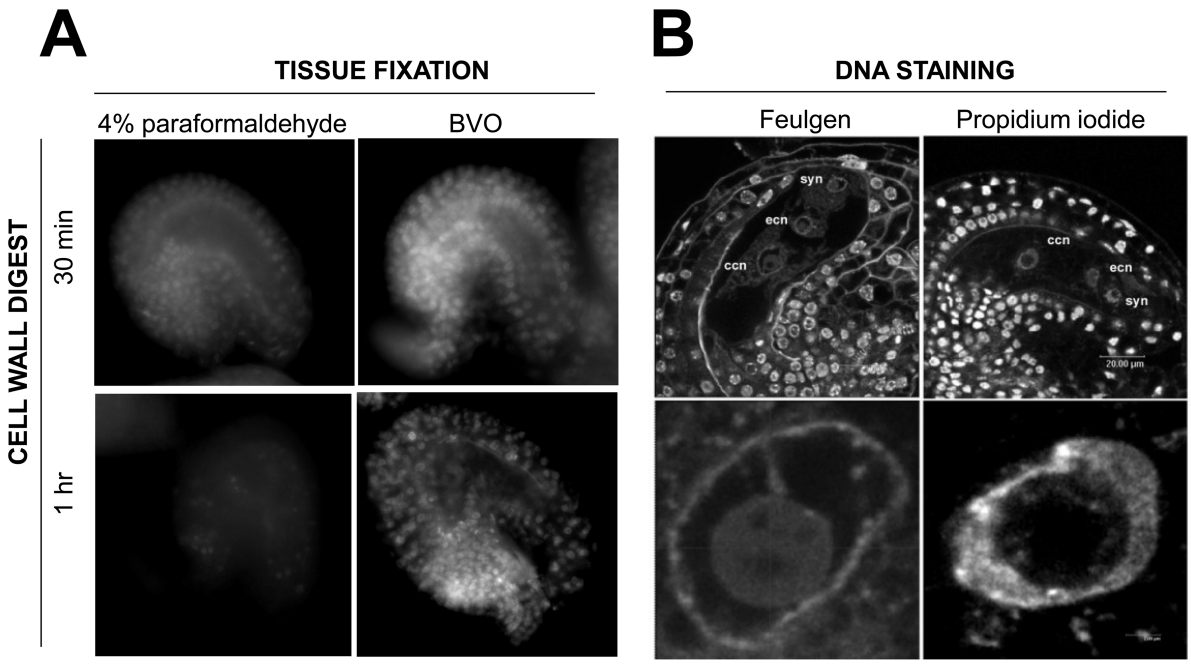


Figure 7



4. Result Chapter II

Chromatin reprogramming in *Arabidopsis* MMC

Specification of spore mother cells (SMCs) in dedicated floral organ is a key step that initiates sexual reproduction in flowering plants, which marks the somatic-to-reproductive cell fate transition. How the reproductive fate is established? It was described that small RNA pathway plays central roles in regulating female spore mother cell (megaspore mother cell, MMC) specification, indicating further chromatin reprogramming in reproductive fate acquisition. It is well established that post-meiotic gametophyte and embryo development in plants is regualted by extensive chromatin reprogramming. Whether chromatin reprogramming occurs before meiosis, that operates MMC fate acquisition, remains unknown.

For this, we analyzed the nuclear organization and chromatin composition in the differeatiating MMC in *Arabidopsis* by the method I described in Chapter I, and found that dynamic chromatin changes take place during the differentiation of MMC, which is likely to play a role in the pluripotent, post-meiotic phase of gametophyte development.

This work was published in *Development* 140, 4008-4019 (She et al., 2013). I contributed to this study with experimental design and interpretation, writing of the manuscript, together with C. Baroux and the other coauthors. Specifically, I conducted the following experiments:

- Analysis of MMC chromatin composition by measuring heterochromatin content, nuclear size, as indicated in Figure 1B, 1C, 1E, 1F.
- Analysis of chromatin modifications in MMC, as described in Figure 3 (except Figure 3F, PolII) and Figure S5.
- Detection of S-phase in differentiating MMC, as in Figure 4A, B, C.
- Analysis of chromatin composition and modifications in MMC at the onset of meiotic Prophase I and functional megaspore (Figure 5A, D, E, G, H, I, J and Figure S7 and S10).
- Functional analyses of chromatin dynamics in MMC (Figure 6E, F, G, H and Figure S4 except Figure S4-E).

In addition, I included the unpublished data concerning further analysis of the mutant where postmeiotic gametophyte is affected (*sdg2*) in the last section of this chapter.

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Chromatin reprogramming during the somatic-to-reproductive cell fate transition in plants

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SUMMARY

The life cycle of flowering plants is marked by several post-embryonic developmental transitions during which novel cell fates are established. Notably, the reproductive lineages are first formed during flower development. The differentiation of spore mother cells, which are destined for meiosis, marks the somatic-to-reproductive fate transition. Meiosis entails the formation of the haploid multicellular gametophytes, from which the gametes are derived, and during which epigenetic reprogramming takes place. Here we show that in the *Arabidopsis* female megaspore mother cell (MMC), cell fate transition is accompanied by large-scale chromatin reprogramming that is likely to establish an epigenetic and transcriptional status distinct from that of the surrounding somatic niche. Reprogramming is characterized by chromatin decondensation, reduction in heterochromatin, depletion of linker histones, changes in core histone variants and in histone modification landscapes. From the analysis of mutants in which the gametophyte fate is either expressed ectopically or compromised, we infer that chromatin reprogramming in the MMC is likely to contribute to establishing postmeiotic competence to the development of the pluripotent gametophyte. Thus, as in primordial germ cells of animals, the somatic-to-reproductive cell fate transition in plants entails large-scale epigenetic reprogramming.

KEY WORDS: *Arabidopsis*, Plant reproduction, Megaspore mother cell, Heterochromatin, Chromatin modifications, Histone variants

INTRODUCTION

In sexually reproducing organisms, gametes are generated by a specific lineage derived from somatic cells that undergo a somatic-to-reproductive cell fate transition (SRT). In mammals, the primordial germ cells (PGCs) differentiate in the embryo at gastrulation stage (Bendel-Stenzel et al., 1998). By contrast, the spore mother cells (SMCs) of flowering plants are formed in the adult plant during floral organ differentiation (Maheshwari, 1950). The female SMC, or megaspore mother cell (MMC), differentiates from nucellar cells within the ovule primordium. The male SMC, or microspore mother cell, develops from the sporogeneous tissue within the anthers. Unlike in animals, the plant products of meiosis (spores) do not directly give rise to functional gametes. Instead, they undergo mitosis to form multicellular structures called gametophytes, which, in turn, give rise to the gametes. In most flowering plants, such as in *Arabidopsis*, the gametophytes are reduced to a small number of cells. The male gametophyte, or pollen, is composed of two sperm cells enclosed within a vegetative cell. The female gametophyte, or embryo sac, is composed of two gametes, termed the egg and central cell, accompanied by accessory cells called antipodals and synergids, the latter of which assist in fertilization. Double fertilization encompasses two fertilization events that produce a totipotent zygote and a nourishing tissue termed the endosperm.

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Plant gametophyte development establishes several cell types with distinct fates over the course of only two to three divisions. For the female gametophyte, which initiates its polarized development as a syncythium, it has been postulated that epigenetic differentiation of the mitotic daughter nuclei might already take place in nuclei before cellularization (Messing and Grossniklaus, 1999; Grant-Downton and Dickinson, 2006). There is a growing body of evidence that gametophyte development is associated with nuclear-scale epigenome remodeling. Dynamic patterns of DNA methyltransferase expression, DNA methylation, and in the distribution of histone variants and histone modifications have been described at discrete stages of embryo sac and pollen development (Ingouff et al., 2007; Schoft et al., 2009; Ingouff et al., 2010; Pillot et al., 2010; Houben et al., 2011; Ibarra et al., 2012; Jullien et al., 2012). Ultimately, an epigenetic dimorphism is established at the level of DNA methylation, histone modifications and their readers, histone variants and transcriptional competence in mature gametophytes, both between the sperm and vegetative cell in the pollen and between the egg and central cell in the embryo sac. This dimorphism is thought to play important functional roles, including the control of transcriptional activity in the egg and early embryo (Pillot et al., 2010) and of transposable elements in the gametes and early embryo, guided by small RNAs (Slotkin et al., 2009; Calarco and Martienssen, 2011; Ibarra et al., 2012). Another wave of reprogramming occurs after fertilization, with the renewal of the repertoire of histone H3 variants in the zygote and the resetting of DNA methylation patterns during the first divisions of the embryo (Ingouff et al., 2010; Jullien et al., 2012).

Thus, two windows of reprogramming have been described during plant reproduction to date: first, during postmeiotic gametophyte development and second, after fertilization during seed development. However, whether reprogramming occurs before meiosis in the SMCs is unknown. In animals, epigenetic reprogramming at the equivalent stage of reproduction, in the PGCs, is crucial for subsequent development. In plants, genetic evidence

indicates that small-RNA-dependent DNA methylation pathways acting in the nucellus surrounding the MMC play a key role both during MMC specification (Olmedo-Monfil et al., 2010; Singh et al., 2011) and later for the initiation of female gametophyte development (Tucker et al., 2012). In addition, SMC differentiation is characterized by elevated transcriptional levels for many of the enzymes that participate in epigenetic regulatory pathways (Berger and Twell, 2011; Schmidt et al., 2011), although their effects on SMC chromatin, as well as their functions in spore and gamete development, remain poorly understood.

Thus, an unresolved question is whether the specification of SMCs, which marks the SRT, coincides with a window of epigenetic reprogramming or whether reprogramming is a sole attribute of postmeiotic development. Here, we analyzed nuclear organization and chromatin composition in the differentiating MMC of *Arabidopsis*. We found highly dynamic chromatin changes coinciding with a slow meiotic S phase, suggesting reprogramming of the epigenetic landscape during MMC specification. Based on the analysis of various mutants, we inferred that these events contribute to the acquisition of the gametophyte fate.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis plants were grown under long-day conditions (16 hours light) at 18-20°C in a plant growth chamber or greenhouse, except for the mutants *ago9-4*, *sgs3-11* and *rdr6-2* (Olmedo-Monfil et al., 2010), which were grown at 23°C in a growth incubator (Percival). The GFP lines shown Fig. 2 and supplementary material Fig. S1 are the following: HTR5-GFP is pHTR5::HTR5-GFP and HTR8-GFP is pHTR8::HTR8-CFP (Ingouff et al., 2010); H2A.Z-GFP is pHTA11::HTA11-GFP (Kumar and Wigge, 2010); HTR12-GFP and GFP-HTR12 (CENH3 lines) are pHTR12::HTR12-GFP (Fang and Spector, 2005) and pCENH3::GFP-CENH3 (Ravi et al., 2011), respectively; LHP1-GFP line is pLHP1::LHP1-GFP (Nakahigashi et al., 2005). A full description of N- and C-terminal fusions of H1 variants with GFP, CFP or RFP is available upon request. In brief, the coding sequence, promoter and 3′UTR (termination) sequences of H1.1 and H1.2 were amplified separately using the primers described in supplementary material Table S8 and subcloned into either pCAMBIA1390 (C-terminal fusions) or a modified pCAMBIA1390 vector where the 35S::HygR resistance cassette has been replaced by a NOS::BAR resistance cassette from pGREENII 029 (Hellens et al., 2000). The EGFP and CFP sequences were subcloned and the RFP-T sequence amplified from the pRFP-T_tag plasmid (Shaner et al., 2008). We analyzed four and six independent lines carrying N- and C-terminal GFP fusions of the H1.1 variant in the *h1.1* mutant background, respectively, three each of N- and C-terminal EGFP fusions to the H1.2 variant, as well as six N-terminal CFP fusions to H1.2 and ten C-terminal RFP fusions to H1.1. For Syringolin A treatment, whole inflorescences were cut and incubated in water (mock) or 100 nM Syringolin A (Groll et al., 2008) in water and placed in the growth chamber for 48 hours before imaging.

Immunostaining in whole-mount ovule primordia

Immunostaining of active PolII was performed as previously described using the anti-RNA Pol II [phospho-S2] antibody (Abcam, ab24758) (Autran et al., 2011). A detailed protocol for immunostaining of histone modifications, H3 and H1 will be published elsewhere. In brief, young carpels were fixed with 1% formaldehyde and 10% DMSO in PBS-Tween (0.1%) before dissection and embedding of the ovule primordia in 5% acrylamide pads on microscope slides. Tissue processing included clarification (methanol/xylene), cell wall digestion and permeabilization before application of the primary, then secondary antibody for 12-14 hours at 4°C. The samples were counterstained with propidium iodide and mounted in Prolong Gold (Invitrogen). Immunostaining efficiency was tested using serial dilutions of the primary antibodies (1:200, 1:500, 1:1000) and the lowest dilution that gave reproducible and homogenous signals was chosen for quantitative imaging (supplementary material Table S7). Control immunodetection in the absence of primary antibody was also performed. The antibodies used are described in supplementary material Table S7.

EdU labeling

A 5-ethynyl-2′-deoxyuridine (EdU)-based assay for S-phase detection was performed as described (Kotogany et al., 2010). Whole inflorescences were incubated in 100 μM EdU solution (Invitrogen, A10044) for 2 hours at 23°C in a plant growth incubator (Percival), then fixed in 4% formaldehyde and 0.1% Triton X-100 in PBS (15 minutes at room temperature), and washed three times in PBS (5 minutes each). Fluorescent labeling of EdU was performed for 30 minutes at room temperature in the dark, followed by three washes in PBS supplemented with 100 ng/ml DAPI. Ovule primordia were then dissected from the carpels and mounted on slides with DAPI in Vectashield (Vector Labs).

Image acquisition and quantitative analyses

Serial images of fluorescent signals in whole-mount ovule primordia were recorded by confocal laser-scanning microscopy with a Leica IRE-SP2 and SP5-R (Leica Microsystems) using a 63× GLY lens (glycerol immersion, NA 1.4). Antibody and DNA signals were acquired sequentially and the volumes were sampled according to the Nyquist rate (2× oversampling). Zoom factor, image geometry, voxel size, scanning speed and averaging were kept identical for the image series in an experiment. Fluorescent signals (GFP, antibody staining, DNA staining) were reported as the intensity sum of voxels per channel in nuclei in 3D-reconstructed images, using manually defined 3D surfaces around MMCs and nucellar nuclei [manual segmentation of nuclear surface using the Surface tool from Imaris software (Bitplane)]. Relative levels of histone H3 and H3 modifications were calculated as a ratio of the intensity sum in the antibody channel over that in the DNA staining channel. For nuclear measurements (as in Fig. 1), the nuclear volume was derived from the statistics of the contours drawn in Imaris, the chromatin volume was derived by subtracting the nucleolus volume (devoid of DNA signal) from that of the nucleus, and heterochromatin content and chromocenter number were calculated as described (Baroux et al., 2007): measurements were made using ImageJ on intensity sum projections from 3D series encompassing (non-overlapping) MMC and nucellus nuclei. The relative heterochromatin fraction (RHF) consisted of the sum of intensity signals in chromocenters (contours defined manually) expressed as a percentage of the total nuclear fluorescence intensity. Quantitative differences were assessed using a Welch's *t*-test (two-tailed).

RESULTS

MMC differentiation is marked by chromatin decondensation and reduction in heterochromatin

The differentiation of the MMC in the ovule primordium marks the onset of female reproductive lineage development. The MMC originates from a single cell in a subepidermal position in the nucellus and is located along the vertical, central axis of the ovule primordium (Fig. 1A) (Schneitz et al., 1995; Yang and Sundaresan, 2000). It undergoes progressive cellular differentiation during primordium growth over a period of 2-3 days before the onset of chromosome condensation during meiotic prophase (Fig. 1A,B). Previous histological studies showed that MMC differentiation is marked by cell enlargement and elongation (Schneitz, 1995) (Fig. 1A) as well as by changes in nuclear and nucleolar size (Cooper, 1937; Schulz and Jensen, 1981; Armstrong and Jones, 2003; Sniezko, 2006) (Fig. 1B-D; supplementary material Table S1). Using non-denaturing whole-mount DNA staining and confocal imaging, we measured a doubling in nuclear volume (Fig. 1C), which appeared to result from both nucleolus enlargement (Fig. 1A) and chromatin decondensation (Fig. 1D). This event coincides with a 60% reduction in heterochromatin content (Fig. 1E) and a decreased number of chromocenters (Fig. 1F). The nuclear organization of the MMC thus markedly differs from that of the surrounding nucellar cells, and nuclear differentiation is visible as

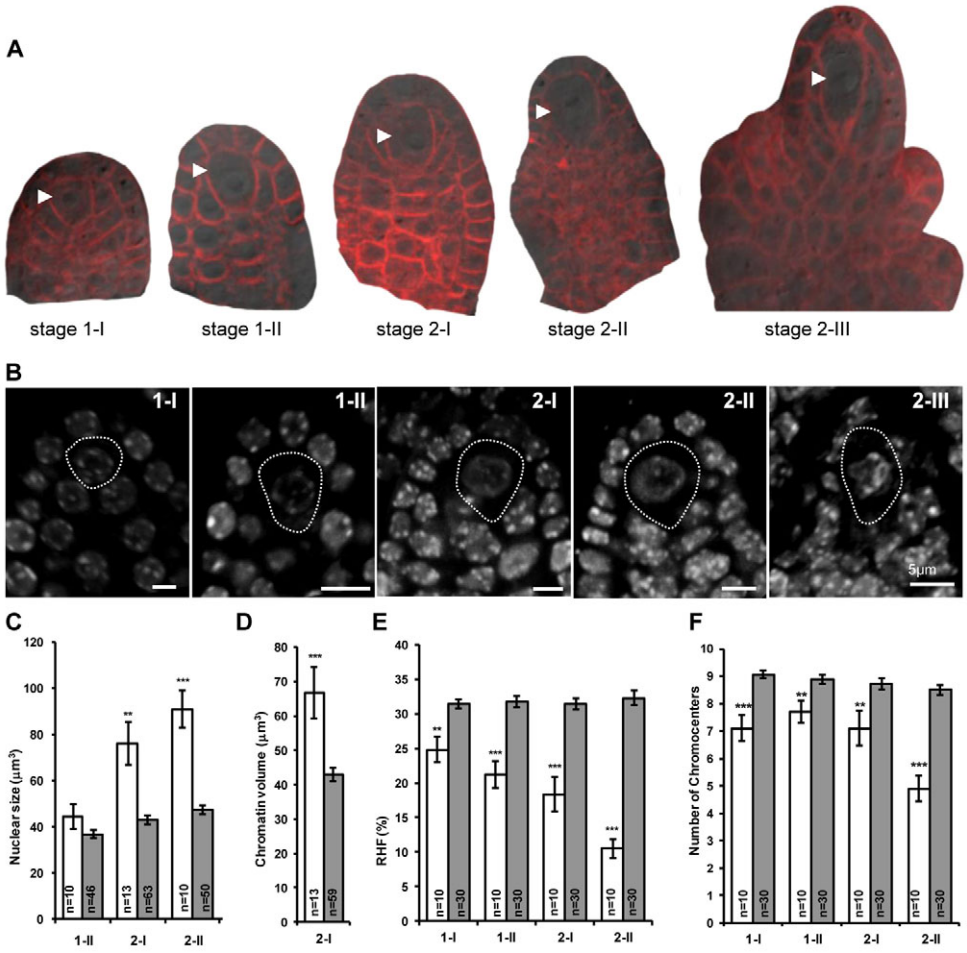

Fig. 1. Nuclear reorganization during MMC specification. (**A**) MMC differentiation in developing *Arabidopsis* ovule primordia at stages 1-I to 2-III (onset of meiosis) as defined by Schneitz et al. (Schneitz et al., 1995). The MMC (arrow) differentiates in a central, subepidermal position in the nucellus. Images are overlays of differential interference contrast (DIC) and FM4-64 counterstaining (red) photographs. (**B**) Whole-mount DNA staining (propidium iodide) allows 3D measurements of nuclear size and heterochromatin organization (C-F) in the MMC (outlined) compared with the surrounding nucellus cells. Partial projections of serial confocal sections are shown. (**C-F**) Quantitative analyses on 3D reconstructions provide measures of nuclear volume (C), chromatin volume (volume of nucleus minus nucleolus) (D), heterochromatin content (E; RHF, relative heterochromatin fraction) and chromocenter number (F). MMC, white bars; nucellus, gray bars. Stages are indicated on the *x*-axis and the number of nuclei analyzed is indicated in each bar (*n*). Error bars indicate s.e.m. Differences between the nucellus and MMC chromatin were assessed using a two-tailed Welch's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001). Detailed quantifications are provided in supplementary material Table S1. Scale bars: 5 µm.

early as stage 1-I, when cell enlargement is also first observed, suggesting an early establishment of an MMC-specific chromatin state.

Histone variants are dynamically exchanged in differentiating MMCs

Linker histones (H1) are essential modulators of chromatin compaction through binding to the linker DNA and the core nucleosome, thereby stabilizing higher-order chromatin structure (Robinson and Rhodes, 2006). The *Arabidopsis* genome encodes three canonical variants (H1.1, H1.2, H1.3), which are broadly expressed during plant development, except for the stress-inducible H1.3 (Ascenzi and Gantt, 1997; Wierzbicki and Jerzmanowski, 2005). To determine whether chromatin decondensation in the MMC correlates with changes in H1 levels, we analyzed the dynamic localization of GFP-tagged variants in developing ovule primordia. We found a sharp decrease of H1.1-GFP and H1.2-GFP levels in MMCs at stage 1-I and undetectable levels at the consecutive stage (Fig. 2A,B). H1.3-GFP was never detected in primordia during MMC differentiation (not shown). The loss of H1.1 and H1.2 is, however, transient: *de novo* incorporation was observed at stages 2-II and 2-III, respectively, and throughout meiosis (supplementary material Fig. S1), consistent with a role of H1 in chromosome condensation. Immunostaining using a novel antibody raised against tobacco H1 confirmed the H1 depletion in the MMC observed with the GFP-tagged lines (supplementary material Fig. S2). Interestingly, the loss of H1 was strongly retarded in the presence of Syringolin A, a potent inhibitor of the plant proteasome (Groll et al., 2008) (Fig. 2C; supplementary material Fig. S3), suggesting the existence of active protein degradation mechanisms controlling H1 depletion from the MMC chromatin. In addition to linker histones, we also analyzed the nuclear distribution of a GFP-tagged variant of LIKE HETEROCHROMATIN PROTEIN1 [LHP1; also known as TERMINAL FLOWER2 (TFL2)], which, in contrast to HP1 in animals, localizes preferentially in euchromatic domains and influences chromatin accessibility (Maison and Almouzni, 2004; Libault et al., 2005; Nakahigashi et al., 2005; Turck et al., 2007). LHP1-GFP signals decreased significantly, although variably, by ~30-60% at stage 2-II (Fig. 2D).

Similarly, we analyzed H3 variants, encoded by the *HISTONE THREE RELATED* (*HTR*) gene family in *Arabidopsis*, fused in frame with fluorescent proteins. HTR12 is the *Arabidopsis* homolog of the centromere-specific variant CENH3 (Talbert et al., 2002). When we examined a C-terminal HTR12-GFP fusion (Fang and Spector, 2005), we observed a drastic depletion from the MMC chromatin at stage 1-II (Fig. 2E). HTR12-GFP signals were faintly recovered just before and at prophase I, although they showed a diffuse distribution at the latter stage (supplementary material Fig. S1). By contrast, the N-terminal GFP-HTR12 fusion (Ravi et al., 2011) showed persistent signals throughout MMC development and, as expected, conspicuous signals in prophase I (Fig. 2F; supplementary material Fig. S1). CENH3/HTR12 reloading has been shown to share structural requirements during male meiosis (as opposed to during mitosis) (Ravi et al., 2011). We thus hypothesized that the MMC chromatin might undergo a rapid turnover of CENH3, whereby the C-terminal

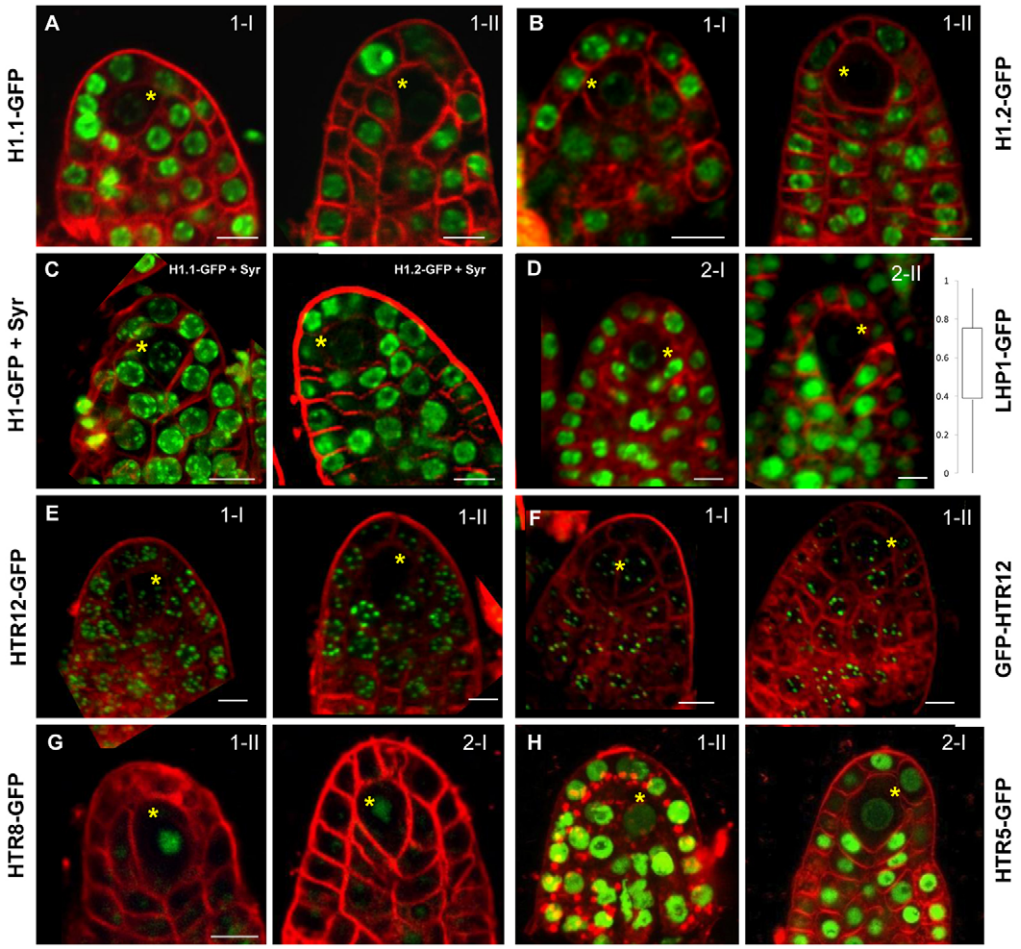

Fig. 2. Dynamics of H1 and H3 histone variants and LHP1 during MMC differentiation. The dynamic nuclear distribution during MMC differentiation of various GFP-tagged chromatin components. (**A-C**) Linker histone variants H1.1 and H1.2 in wild-type MMCs (A,B) and in MMCs exposed to 100 nM Syringolin A, a potent inhibitor of the plant proteasome, for 48 hours (C). (**D**) Chromatin-associated protein LHP1. (**E**,**F**) Centromeric H3 variant CENH3/HTR12 as N-terminal (E) or C-terminal (F) fusions. (**G**,**H**) H3.3 variants HTR8 (G) and HTR5 (H). Green, GFP fluorescence; red, FM4-64 fluorescence. Asterisks indicate the MMC. For LHP1 (D), reduced levels in stages 2-I and 2-II were revealed upon signal quantification on 3D reconstructions. The box plot shows the fluorescence intensity ratios between MMC (n=12) and nucellus (n=44) chromatin (whiskers are upper and lower quartiles). The dynamic distribution of these GFP-tagged chromatin proteins throughout the entire phase of MMC development, meiosis and the FMS is shown in supplementary material Fig. S1. Scale bars: 5 μm.

fusion was improperly reloaded in the MMC following eviction, as has been described in male meiocytes (Ravi et al., 2011). By contrast, we also observed HTR8 and HTR5 (Ingouff et al., 2010) in the MMC (Fig. 2G,H), two H3.3 variants that are usually associated with transcriptional competence (Ahmad and Henikoff, 2002; Mito et al., 2005; Wollmann et al., 2012). This also indicates that loss of GFP signals in the MMC observed for the other histone-tagged variants is not an artifact.

Finally, changes in the repertoire of histone variants also encompassed H2A.Z, a labile variant that marks poised genes involved in rapid environmental responses (Deal and Henikoff, 2010; Kumar and Wigge, 2010; Coleman-Derr and Zilberman, 2012). We observed that a GFP-tagged HTA11/H2A.Z variant was evicted from the MMC chromatin as early as stage 2-I, and then reincorporated prior to prophase I (supplementary material Fig. S1).

Collectively, the depletion of linker histones H1.1, H1.2, LHP1 and H2A.Z is consistent with a global pattern of chromatin decondensation in the MMC. The dynamics of HTR12/CENH3, of the HTR5 and HTR8 H3.3 variants and of HTA11/H2A.Z indicate that core nucleosomes are remodeled, illustrating a global yet specific chromatin reprogramming during MMC differentiation.

MMC chromatin differs from that of surrounding somatic cells by distinct levels of histone H3 and H4 modifications and active RNA polymerase II

The changes we observed in nuclear organization and chromatin condensation in MMCs suggest the establishment of an open, permissive chromatin state. This state is usually associated with, and mediated by, biochemical modifications of specific amino acid residues of nucleosomal histones. We thus analyzed the relative levels of histone modifications associated with either a permissive (H3K4me2, H3K4me3) or repressive (H3K27me3) environment of euchromatic regions in plant nuclei (Fuchs et al., 2006). We performed immunostaining on whole-mount ovule primordia and compared MMC chromatin with that of neighboring nucellar cells (Fig. 3; supplementary material Table S2). In particular, we determined the chromatin state of MMCs at the end of their differentiation, at stage 2-II and just prior to prophase I. Whereas H3 levels, which were used as a control, were similar in both cell types (Fig. 3A), there was a 2.7-fold enrichment of H3K4me3 levels and a 50% reduction of H3K27me3 levels in the MMC relative to cells of the nucellus (Fig. 3B,C). H3K27me3 levels were not affected in mutants lacking RELATIVE OF EARLY FLOWERING6 (REF6) activity, an enzyme that catalyses H3K27me2/me3 demethylation (Lu et al., 2011) (supplementary material Fig. S4), indicating that loss of H3K27me3 in the MMC is either passive or mediated by another, as yet unknown, histone demethylase. Similarly, in mutants lacking TRITHORAX-RELATED1 (ATX1) activity, which catalyses H3K4 methylation and counteracts H3K27me3 repression (Alvarez-Venegas et al., 2003; Saleh et al., 2007), H3K4me3 levels in the MMCs were unaffected (supplementary material Fig. S4), suggesting the involvement of other SET-domain enzymes of redundant function with ATX1 (Thorstensen et al., 2011).

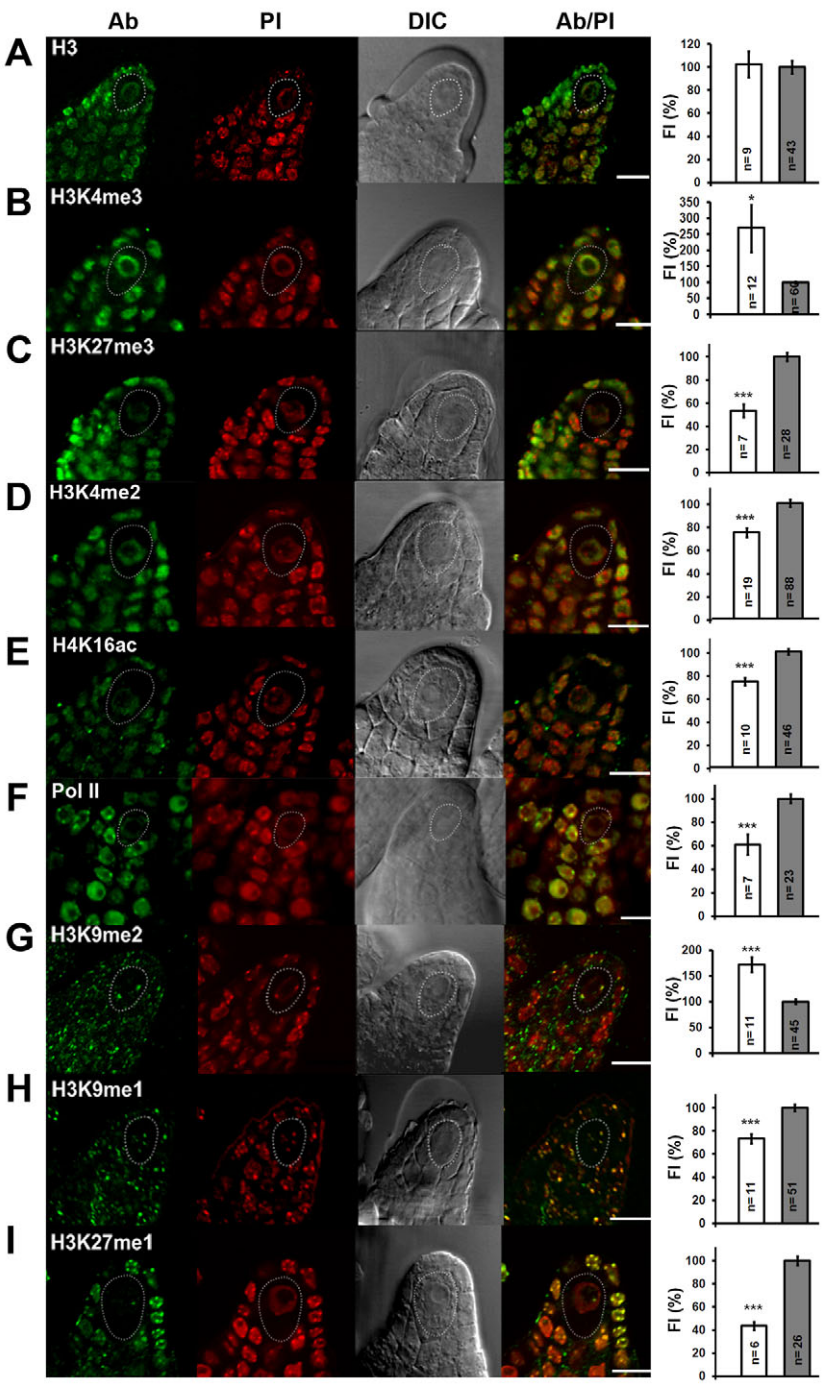

Fig. 3. The chromatin of the MMC is epigenetically distinct from that of the surrounding nucellar cells. (**A-I**) Global levels of H3 (A), modified H3 (B-D,G-I) and H4 (E) and active (Ser2 phosphorylated) PolII (F) were determined in the MMC and surrounding nucellar cells by whole-mount immunostaining in ovule primordia at stage 2-II. Representative images are shown for the antibody (Ab), DNA (propidium iodide, PI), transmitted light (DIC), and antibody signal overlaid with the DNA signal (Ab/PI). The relative fluorescence intensity in each channel was determined in 3D reconstructions in individual MMCs and nucellus nuclei. Bar charts show the average ratio Ab/PI relative to the nucellus (100%). White, MMC; gray, nucellus. The number of nuclei measured is indicated in each bar (*n*). Error bars indicate s.e.m. **P*<0.05, ****P*<0.001 (two-tailed Welch's *t*-test). Detailed quantifications at stage 2-II are provided in supplementary material Table S2 and the dynamics over the stages 1-II to 2-II are presented in supplementary material Fig. S5 and Table S3. Scale bars: 10 μm.

The histone marks found in MMCs are consistent with the establishment of a permissive chromatin environment. However, we also observed that the levels of H3K4me2 are reduced by 30% (Fig. 3D), as are the levels of H4K16ac, which correlates with active transcription (Tian et al., 2005; Jang et al., 2011; Vaquero-Sedas et al., 2011) (Fig. 3E). Furthermore, the presence of active RNA polymerase II (Ser2-phosphorylated RNA PolII) was significantly reduced (Fig. 3F), suggesting low transcriptional competence in the MMC.

Heterochromatin in the chromocenters of the *Arabidopsis* nucleus consists of centromeric and pericentromeric repeats, including transposons and rDNA sequences enriched in H3K9me1, H3K9me2, H3K27me1, H3K27me2, H4K20me1 and methylated DNA (Fransz et al., 2006). H3K9me2 is a hallmark of constitutive heterochromatin, yet is dispensable for its formation (Jasencakova et al., 2003). In dedifferentiated cells, such as protoplasts, or leaf cells lacking a DNA methylation maintenance function mediated by DECREASE IN DNA METHYLATION1 (DDM1), chromocenters are entirely disassembled whereby H3K9me2 immunostaining signals and centromeric repeats are redistributed (Jasencakova et al., 2003; Tessadori et al., 2007). In MMCs, by contrast, although the reduction in heterochromatin is comparable to that of dedifferentiated cells, H3K9me2 remained localized at conspicuous, although less numerous, chromocenters (Fig. 3G). This suggests that a fraction of sequences formerly associated with chromocenters were dispersed and lost H3K9me2

enrichment. Furthermore, the 1.6-fold enrichment of global H3K9me2 levels in MMC chromatin compared with nucellar cells suggests a reinforcement of heterochromatin silencing at sequences present in chromocenters. Increased H3K9me2 levels seem to occur at the expense of H3K9me1, which is present at reduced levels in MMC chromatin (Fig. 3H), possibly as a result of conversion to the dimethylated form by SUVR4 (Veiseth et al., 2011). Furthermore, the increase in H3K9me2 levels seems highly specific, as H3K27me1, another mark typically enriched at chromocenters, was almost absent from MMC heterochromatin at stage 2-II, with a 60% depletion relative to nucellar cells (Fig. 3I).

Clearly, MMC specification is accompanied by the establishment of a highly distinct chromatin configuration compared with that of the nucellus from which the MMC is derived. The MMC chromatin state is transcriptionally more permissive, yet has attenuated transcriptional competence. Also, it harbors a reduced heterochromatin fraction, yet is enriched in marks typical of silenced chromatin.

Reprogramming of chromatin modifications is gradual and partially synchronous with meiotic S phase, uncoupling heterochromatin and euchromatin replication

The chromatin state at stage 2-II is established gradually and asynchronously among histone modifications. Typically, H1 eviction precedes all other changes sequentially affecting heterochromatin and euchromatin, with the heterochromatic mark H3K9me2 increasing already at stage 1-II, whereas the euchromatic marks H3K27me3 and H3K4me3 change significantly only at later stages (2-II) (supplementary material Fig. S5, Table S3). To determine whether these distinct dynamics relate to a specific phase of the cell cycle (G1, S or G2), we characterized the meiotic S phase in the MMC. We quantified the DNA content in the MMC and compared it with that of three epidermal cells at the very tip of the nucellus (Fig. 4A; supplementary material Table S4). The DNA content progressively increased from stage 1-I to stage 2-II, indicating a slow genome replication occurring over several days. Consistent with this, *de novo* nucleotide incorporation, using EdU labeling (2-hour pulse), was observed as early as stage 1-I and lasted until stage 2-II (Fig. 4B,C). We also observed the continuous presence of a GFP-tagged variant of ORIGIN REPLICATION COMPLEX2 (ORC2-GFP) (Ngo et al., 2012) throughout meiotic S phase; this might indicate either progressive marking of early versus late replication origins or a role in establishing sister chromatid cohesion (MacAlpine et al., 2010) (Fig. 4D).

Thus, at the stage of H1 eviction (stage 1-I), the MMC has already engaged in DNA replication. However, the global loss of linker histones is unlikely to be a requirement for DNA replication as H1.1 remains detectable during the S phase of mitotic cell cycles (supplementary material Fig. S6). Interestingly, *de novo* EdU incorporation was predominantly found in heterochromatic chromocenters for MMCs at stage 1-I and 1-II, and in euchromatin for MMCs at stage 2-II (Fig. 4B,C; supplementary material Table S4). This suggests that the replication of cytologically detectable heterochromatin regions precedes that of most of the euchromatin during the meiotic S phase of female meiocytes in *Arabidopsis*. Furthermore, this observation raises the possibility that chromatin dynamics in heterochromatin (e.g. H3K9me2) and euchromatin (e.g. H3K27me3 and H3K4me3) might be coupled with the asynchronous replication of some heterochromatin and euchromatin regions, respectively.

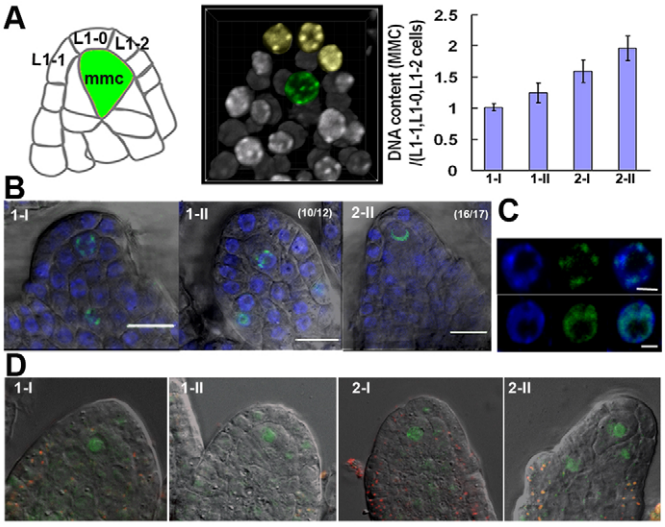

Fig. 4. A slow meiotic S phase in the MMC uncouples replication of heterochromatin and euchromatin. (**A**) Quantification of the DNA content in MMCs compared with that of the epidermal cells covering them (L1-1, L1-0, L1-2) indicates a progressive increase from G1 (stage 1-I) to G2 (stage 2-II) over several days. For each stage, *n*=10 MMCs and *n*=30 L1 cells. Bar chart shows the average ratio of DNA fluorescence intensity in MMC/nucellus; error bars indicate s.e.m. Detailed quantifications are given in supplementary material Table S4. (**B**,**C**) A 2-hour pulse of EdU incorporation reveals *de novo* DNA synthesis as early as stage 1-I predominantly in heterochromatin, and until stage 2-II when replication is essentially in euchromatin. The ratios (B) indicate the number of specific patterns/total observations: 10/12 primordia at stages 1-I and 1-II showed EdU signals enriched in heterochromatin foci; 16/17 primordia at stages 2-I and 2-II showed euchromatin signals. (C) A detailed view of the specific heterochromatin and euchromatin enrichment patterns in MMC nuclei at early (top) and late (bottom) stages. (**D**) Persistent nuclear localization of the ORC2 subunit (green) in MMCs throughout meiotic S phase. Scale bars: 10 μm.

Meiosis and selection of the functional megaspore entail additional and specific chromatin dynamics

Chromosome condensation marks the onset of the first meiotic prophase and is observed at stage 2-III of ovule development (Fig. 1). Consistent with their expected functions during chromosome condensation and segregation, respectively, the expression of H1.1 and H1.2 and of CENH3 is restored during meiosis (supplementary material Fig. S1). Furthermore, the increasing and decreasing trend of H3K9me2 and H3K27me3 signals, respectively, during MMC differentiation became more pronounced at prophase I, while H3K27me1 signals appeared stronger than at stage 2-II (supplementary material Fig. S7). Although the rapidly changing meiotic chromosomes render precise quantification difficult, meiosis clearly entails further changes in chromatin modifications.

Female meiosis produces four haploid spores, three of which degenerate while the surviving one, the functional megaspore (FMS), acquires a pluripotent fate; the FMS will form the multicellular gametophyte within which the gametes differentiate. To determine whether the chromatin state of the FMS resembles or differs from that of the MMC, we analyzed the same histone variants and modifications as above (Fig. 5; supplementary material Table S5, Figs S1, S8). Interestingly, the FMS chromatin

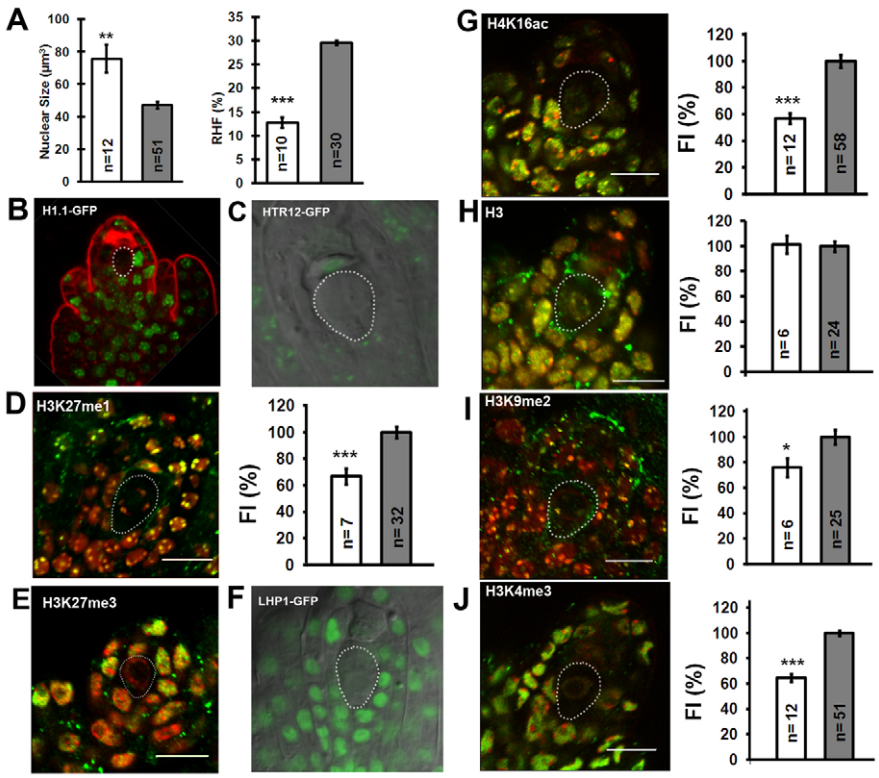

Fig. 5. Nuclear organization and chromatin state of the FMS resembles that of the MMC, but with specific hallmarks. (**A-J**) Nuclear organization and chromatin state of the FMS (the meiotic product giving rise to the embryo sac) was analyzed as for the MMC in Figs 1-3. The chromatin of the FMS largely recapitulates the state established in the MMC prior to meiosis, despite transient dynamic changes of some histone variants during meiosis (supplementary material Fig. S1). Exceptions are the histone modifications H3K9me2 and H3K4me3, which are decreased relative to the levels in the nucellus. The FMS is outlined. Error bars indicate s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001 (two-tailed Welch's *t*-test). Detailed quantifications are provided in supplementary material Table S5. Scale bars: 10 µm.

recapitulated the majority of characteristics observed in MMCs. This included decondensed chromatin, reduced heterochromatin content (Fig. 5A), a notable absence of H1.1 and H1.2 (Fig. 5B; supplementary material Fig. S1), undetectable levels of CENH3, strongly reduced levels of H3K27me1 (Fig. 5C,D), as well as undetectable levels of LHP1 and H3K27me3 (Fig. 5E,F). Similar to observations in the MMC, FMS chromatin shows low levels of H4K16ac (Fig. 5G) and active PolII, indicating low transcriptional activity, at least for the stage that we observed, just following FMS selection (supplementary material Fig. S8). At this stage, replication for subsequent gametogenesis has started (supplementary material Fig. S8). Furthermore, the detection of similar levels of H3 in the FMS and nucellar cells (Fig. 5H) confirmed that the decreased levels of immunostaining that we observed are not due to technical limitations. However, the FMS also exhibits specific, postmeiotic chromatin changes. The repressive mark H3K9me2 and the permissive mark H3K4me3, which were enriched in the MMC, were reduced in FMS chromatin compared with that of surrounding somatic cells (Fig. 5I,J).

Collectively, these results indicate that not only meiosis but also FMS selection entail additional chromatin reprogramming processes that follow both similar and specific trends compared with MMC differentiation.

Chromatin reprogramming in the MMC is likely to contribute to establishing competence for the postmeiotic fate

In wild-type *Arabidopsis* only one MMC is specified in the nucellus. The ARGONAUTE family member ARGONAUTE9 (AGO9) plays an essential role in restricting to a single MMC the number of reproductive lineage cells in each ovule via a small-RNA-dependent pathway. In the absence of AGO9, additional enlarged germline cells form ectopically and initiate the postmeiotic, gametophytic program (Olmedo-Monfil et al., 2010). To determine whether the chromatin dynamics that we observed in wild-type MMCs and FMSs are linked to the SRT, we looked at several chromatin markers in these supernumerary germline precursor cells. Similar to MMCs, the chromatin of these ectopic cells is devoid of H1.1 and H1.2 (Fig. 6A,B), whereas it retains HTR5 and HTR8 (Fig. 6C,D). The loss of both linker histone variants was confirmed in mutants affecting other components of this regulatory pathway [*suppressor of gene silencing3* (*sgs3*) and *rna-dependent rna polymerase6* (*rdr6*) mutants; supplementary material Fig. S9]. Furthermore, in *ago9* mutants, these supernumerary germline precursor cells also showed reduced levels of heterochromatic H3K27me1 (Fig. 6E; supplementary material Table S6), a hallmark of both the MMC and FMS chromatin state. Similarly, we also observed a drastic reduction of the euchromatic repressive modification H3K27me3 in these cells in a comparable manner to that in wild type (Fig. 6F; supplementary material Table S6). Because ectopic cells in *ago9* mutant ovule primordia do not initiate meiosis but instead directly differentiate into FMS (Olmedo-Monfil et al., 2010), these observations strongly support the idea that chromatin reprogramming constitutes a cell fate marker of the SRT.

We also analyzed mutant MMCs lacking the activity of SET DOMAIN GROUP2 (SDG2), one of several enzymes responsible for H3K4me3 deposition in *Arabidopsis* (Berr et al., 2010; Guo et al., 2010). In homozygous mutants, ovules are sterile and germline development typically shows a postmeiotic arrest at the FMS stage (Berr et al., 2010). Yet, we have shown that global levels of H3K4me3 increase in the MMC whereas they decrease in the FMS, suggesting that SDG2 might function before, rather than after, meiosis. Consistent with this hypothesis, the relative enrichment of H3K4me3 levels in *sdg2* MMCs only reached 60% of that in the wild type (Fig. 6G; supplementary material Table S6). Incidentally, detectable H3K4me3 in *sdg2* mutant MMCs suggests the activity of other H3K4 methyltransferases encoded in the *Arabidopsis* genome (Thorstensen et al., 2011). We observed progressive chromosome

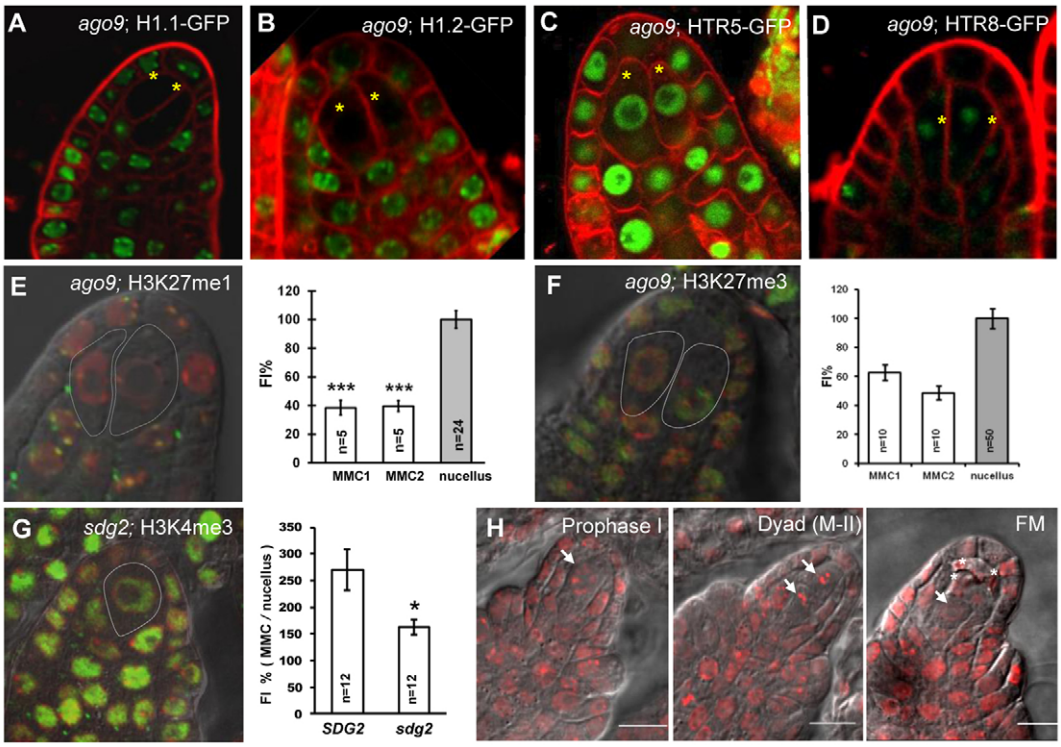

Fig. 6. Chromatin reprogramming in the MMC establishes competence for the postmeiotic developmental fate. (**A-F**) Ovule primordia lacking AGO9 activity show ectopic differentiation of germline precursor cells next to the MMC, expressing an ameiotic gametophyte developmental fate (Olmedo-Monfil et al., 2010). These cells recapitulate key events of chromatin reprogramming typical of MMCs: H1.1 and H1.2 depletion (A,B), expression of HTR5 and HTR8 variants (C,D), reduction of heterochromatin content and associated H3K27me1 (E) and reduction of the repressive euchromatic modification H3K27me3 (F). Quantifications as in Fig. 3. The analysis of H1.1-GFP in additional mutants of the AGO9 pathway are shown in supplementary material Fig. S9. (**G**,**H**) Ovule primordia lacking SDG2 activity show lower levels of H3K4me3 in the MMC relative to the nucellus, compared with in wild type. Yet this deficiency does not impair meiosis (H) (supplementary material Fig. S10), but compromises postmeiotic development (Berr et al., 2010). Error bars indicate s.e.m. **P*<0.05, ****P*<0.001 (two-tailed Welch's *t*-test). Detailed quantifications are given in supplementary material Table S6. Arrows in H indicate the MMC in prophase I, the two cells at the dyad stage and the four cells of the tetrad with FMS; asterisks indicate degenerated spores. Scale bars: 10 µm.

condensation at prophase I, as well as dyads and tetrads in *sdg2* developing ovules, suggesting that the substantial reduction in global H3K4me3 in *sdg2* MMCs did not affect meiosis (Fig. 6H; supplementary material Fig. S10) but rather prevented the FMS from initiating postmeiotic development (Berr et al., 2010) (our observations, not shown).

Altogether, our analyses of two antagonist mutants that ectopically express (*ago9*) or, by contrast, are impaired (*sdg2*) in the female gametophyte fate indicate that chromatin reprogramming is linked to, and is likely to contribute to, the acquisition of competence for the postmeiotic development of the female germline.

DISCUSSION

The data reported here suggest that, in the *Arabidopsis* ovule, the SRT is marked by extensive nuclear reorganization with drastic changes in chromatin condensation, composition and histone modification, including heterochromatin content and distribution. Analyses of mutants affecting megaspore differentiation indicate that these events contribute to establishing competence for the postmeiotic, gametophyte fate. The data reported here suggest that, in the *Arabidopsis* ovule, the SRT is marked by extensive nuclear reorganization with drastic changes in chromatin condensation, composition and histone modification, including heterochromatin content and distribution (Fig. S11).

Mechanisms of chromatin reprogramming

Chromatin reprogramming in the MMC is gradual and occurs in two consecutive phases characterized by: (1) early and rapid events including H1 and H2A.Z eviction, CENH3 turnover, increased levels of H3K9me2; and (2) late changes including decreased levels of H3K27me1, H3K9me1, H3K27me3, LHP1, H4Kac16 and PolII activity, while H3K4me3 levels increase. Interestingly, these two phases take place during a long meiotic S phase, during which we found a preferential replication of heterochromatin at early S phase and preceding that of euchromatin. Although late heterochromatin replication has been reported in male meiocytes of other plant species (Holm, 1977; Greer et al., 2012; Higgins et al., 2012), early replication is not uncommon and was found in male mammalian meiocytes (Latos-Bielenska and Vogel, 1992) or during animals mitosis [(Kim et al., 2003) and references therein].

The two phases of chromatin reprogramming might be partially coupled to the two phases of meiotic replication, raising the possibility of replication-dependent processes. For instance, the *de novo* incorporation of non-modified histones might partially contribute to the reduced H3K27me3:DNA signal ratio (supplementary material Fig. S4). However, the decreased absolute levels of H3K27me3 signal also implicate active demethylation. Yet a loss-of-function mutation in *REF6*, which encodes the major H3K27me3 demethylase (Lu et al., 2011), did not affect this process

(supplementary material Fig. S4), suggesting the involvement of as yet unknown alternative enzymes.

Increased levels of H3K9me3 at early stages of MMC differentiation are also likely to involve histone methyltransferase activity recruited at early S phase. When we examined mutants lacking SUVAR3-9 HOMOLOG4 (KRYPTONITE) activity (Lindroth et al., 2004), we found no cytologically detectable levels of H3K9me2 in ovule primordia (n=24; supplementary material Fig. S4), as has been reported for mature ovules (Autran et al., 2011). However, these mutants exhibit normal fertility, suggesting the involvement of factors that act redundantly with H3K9me2 in heterochromatin silencing (Thorstensen et al., 2011).

H4K3me3 chromatin modification is catalysed by several enzymes, including *Arabidopsis* ATX1 and SDG2, as well as other potentially active SET or ATRX proteins (Alvarez-Venegas and Avramova, 2002). Whereas we found no changes in H3K4me3 levels in *atx1* mutant primordia (supplementary material Fig. S4), SDG2 contributed to H3K4me3 dynamics in the MMC (Fig. 6 and discussed below).

Finally, genome-wide eviction of H1 and H2A.Z histone variants occurs prior to euchromatin replication, suggesting an active, replication-independent process, yet possibly coordinated by cell cycle regulators. In animals, CDK2-mediated phosphorylation of histone H1 during the mitotic S phase destabilizes H1-chromatin interactions resulting in a more open chromatin structure (Contreras et al., 2003). A similar process is likely to be in place in plants, whereas a CDK2 type of activity could possibly contribute to H1 phosphorylation in wheat male meiocytes (Greer et al., 2012). In addition, H1 binding is modulated by the histone chaperone NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1) (Kepert et al., 2005). *Arabidopsis* plants lacking NAP1;1-3 activity [triple mutant (Liu et al., 2009)] or the activity of the NAP1-related proteins NRP1 and NRP2 [double mutant (Zhu et al., 2006)] show normal eviction of both H1.1 and H1.2 in the MMC and reincorporation at meiosis (supplementary material Fig. S4). Clearly, however, a proteasome-mediated degradation process appears to contribute to eliminate (probably unbound) H1 from the MMC chromatin (Fig. 2; supplementary material Fig. S3). Further investigations are thus required to identify the factors controlling H1 dynamics in the MMC, a precocious event that is likely to be crucial for epigenetic reprogramming (see below).

Chromatin reprogramming in the MMC and meiosis

The SRT is intimately linked with the transition from a mitotic to a meiotic cell cycle program. Chromatin reprogramming in the MMC could thus potentially serve several meiotic functions: in regulating entry to the meiotic cell cycle, in meiotic progression, or both.

A few reproductive mutants in maize and *Arabidopsis* initiate SMC differentiation but fail to enter meiosis and produce non-reproductive mitotic cells (Pawlowski et al., 2007), showing that SMC specification can be uncoupled from the meiotic program. In the budding yeast *Saccharomyces cerevisiae*, depletion of the linker histone Hho1 is a prerequisite to instruct entry to meiosis through derepression of meiotic genes (Bryant et al., 2012). H1 depletion in the MMC occurs as early as stage 1-I of ovule primordia development, coinciding with the onset of the meiotic S phase. This event could thus contribute to instructing entry into meiosis in MMCs. However, H1 depletion is likely to have additional functions because ectopic *ago9* MMCs also undergo this event yet avoid meiosis and initiate gametophyte development instead (Olmedo-Monfil et al., 2010).

The meiotic cell cycle is initiated at interphase, during which a typically long S phase takes place in most organisms studied so far, and deserves preparatory functions to meiotic execution (Bennett, 1977). The end of DNA replication entails the establishment of sister chromatid cohesion, a prerequisite for homologous chromosome pairing and synapsis enabling meiotic recombination during prophase I (Osman et al., 2011). It was recently suggested that H1 destabilization upon CDK2-mediated phosphorylation during the meiotic S phase might contribute to heterochromatin decondensation, facilitating, in turn, sister chromatid cohesion in wheat male meiocytes (Greer et al., 2012). In addition, perturbation of H1 stoichiometry in tobacco flowers induced aberrant male meiosis involving incorrect chromosome pairing and segregation (Prymakowska-Bosak et al., 1999). Thus, H1 dynamics in *Arabidopsis* MMCs committed to meiosis (depletion at early S phase and reloading at early prophase I) might reflect similar roles. Furthermore, additional chromatin modifiers are essential to the execution of meiotic events following meiotic S phase (Tiang et al., 2012). For instance, DNA methylation and histone H4 acetylation events contribute to chiasma distribution and frequency in *Arabidopsis* (Perrella et al., 2010; Yelina et al., 2012). Whether H3K9 and H3K4 methylation also contribute to the formation and repair of double-strand breaks, which underlies genetic recombination, in plant as in animal meiosis (Ivanovska and Orr-Weaver, 2006; Borde et al., 2009) remains to be determined. A speculative role for chromatin reprogramming in the MMC could thus lie in the establishment of an epigenetic landscape that is instructive for further chromatin dynamics and subsequent meiotic events (Tiang et al., 2012).

Chromatin reprogramming and epigenetic resetting

Besides the possible contribution to meiotic execution mentioned above, chromatin reprogramming clearly plays a role in establishing the postmeiotic, gametophyte fate. This conclusion is based on the analysis of two antagonistic mutants in which gametophyte development is either ectopically expressed and meiosis is avoided (*ago9*) or is impaired while meiosis progresses apparently normally (*sdg2*). We thus propose that chromatin reprogramming underlies a process of epigenetic reprogramming.

From a developmental perspective, SMCs are the functional equivalent of animal PGCs. During development, PGCs undergo a genome-wide and complex chromatin reprogramming, including nuclear size increase, loss of heterochromatic chromocenters, depletion of somatic linker histones and chromatin decondensation, redistribution of HP1, reduction in H3K9me2, H3K9ac and H3K27me3 and histone replacements (Hajkova et al., 2008; Seki et al., 2005; Mansour et al., 2012). These events take place during a proliferative phase of PGCs and part of the chromatin dynamics may be coupled with the cell-cycle stage (Kagiwada et al., 2012). Interestingly, chromatin dynamics in MMCs share many features with that of animal PGCs. Epigenetic reprogramming in PGCs has multiple roles, including preparation for meiosis, erasure of imprints and epimutations, and the removal of epigenetic barriers to pluripotency, thereby resetting the ‘ground state’ of the epigenome (Hajkova, 2011; Hackett et al., 2012). In particular, H3K27me3 demethylation, H1 depletion and DNA demethylation are crucial for enabling pluripotency (Terme et al., 2011; Hackett et al., 2012; Mansour et al., 2012). Similarly, H3K27me3 reprogramming, chromatin decondensation and reduction in H1 and LHP1 are considered as hallmarks of plant cell dedifferentiation towards pluripotency (Zhao et al., 2001; Williams et al., 2003; Tessadori et

al., 2007; Alatzas et al., 2008; He et al., 2012). The analysis of *ago9* ectopic germline precursor cells, which exhibit an ameiotic, gametophyte fate, further indicate that H1 depletion as well as H3K27me3 reduction in the MMC contribute to establishing a gametophyte fate, which also involves pluripotent development. Further evidence for this hypothesis is provided by the overexpression of H1 genes in the nucellus of maize hybrids developing ameiotic, unreduced gametophytes (Garcia-Aguilar et al., 2010). Moreover, the analysis of plants lacking SDG2 activity uncovered a postmeiotic role for H3K4me3 deposition in the MMC. In *sdg2* MMCs, H3K4 methylation is not fully compromised and meiotic progression appears normal at the cytological level, yet FMSs are not competent to resume gametophyte development (Berr et al., 2010) (our observations). Thus, we propose that reprogramming of the H3K4me3 landscape at the end of the meiotic S phase contributes to the transcriptional activation of genes relevant for the pluripotent development of the female gametophyte. Consistent with this hypothesis, the MMC transcriptome was found to express genes involved in gametophyte but also in early embryo development, suggesting potential long-term relevance of reprogramming events in the MMC (Schmidt et al., 2011).

The accepted view is that DNA methylation patterns are relatively stable during plant reproduction (Jullien and Berger, 2010), allowing for transgenerational inheritance of epigenetic states (Paszkowski and Grossniklaus, 2011; Saze, 2012). However, H1 stoichiometry in the plant nucleus, besides its impact on the structural organization of chromatin, is likely to influence DNA methylation patterns as it does in both plant and animal somatic cells (Fan et al., 2005; Wierzbicki and Jerzmanowski, 2005; Yang et al., 2013; Zemach et al., 2013). H2A.Z depletion in the MMC further increases the potential for reprogramming of DNA methylation because these two marks are mutually exclusive (Zilberman, 2008). Profiling of methylated sites in different contexts (CG, CHG and CHH) (Vaillant and Paszkowski, 2007) will be required to resolve the extent to which H1 and H2A.Z dynamics influence DNA methylation patterns during MMC specification. How this global reprogramming event would be compatible with the transgenerational inheritance of epigenetic states remains to be determined (Paszkowski and Grossniklaus, 2011; Saze, 2012). However, the tools necessary for such investigations are not currently available: single-cell epigenome profiling in MMCs is hindered by the high cellular dilution of MMCs within floral tissues (Wuest et al., 2013), and cytogenetic mapping of DNA methylation provides insufficient resolution with signals preferentially located at the periphery of centromeres (Fransz et al., 2006; Zhang et al., 2008) and does not resolve the sequence context of DNA methylation.

Although further technological improvements and investigations are required to disentangle the short- and long-term developmental impact of the different events affecting the MMC epigenome, we propose that global chromatin decondensation and H1 eviction in MMCs might allow an initial relaxation of the chromatin structure that is compatible with large-scale reprogramming of H3K27me3 and H3K4me3 patterns and possibly also DNA methylation. Collectively, these events are likely to contribute to establishing competence for the pluripotent, postmeiotic fate in the female gametophyte. Epigenome profiling in the MMC remains the next challenge to further define these reprogramming events.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
The experiments were designed by W.S., D.G., K.R., A.J. and C.B., and carried out by W.S., D.G., K.R., M.W., M.P., M.K. and C.B. W.S., D.G., K.R., A.J. and C.B. wrote the manuscript.

Supplementary material
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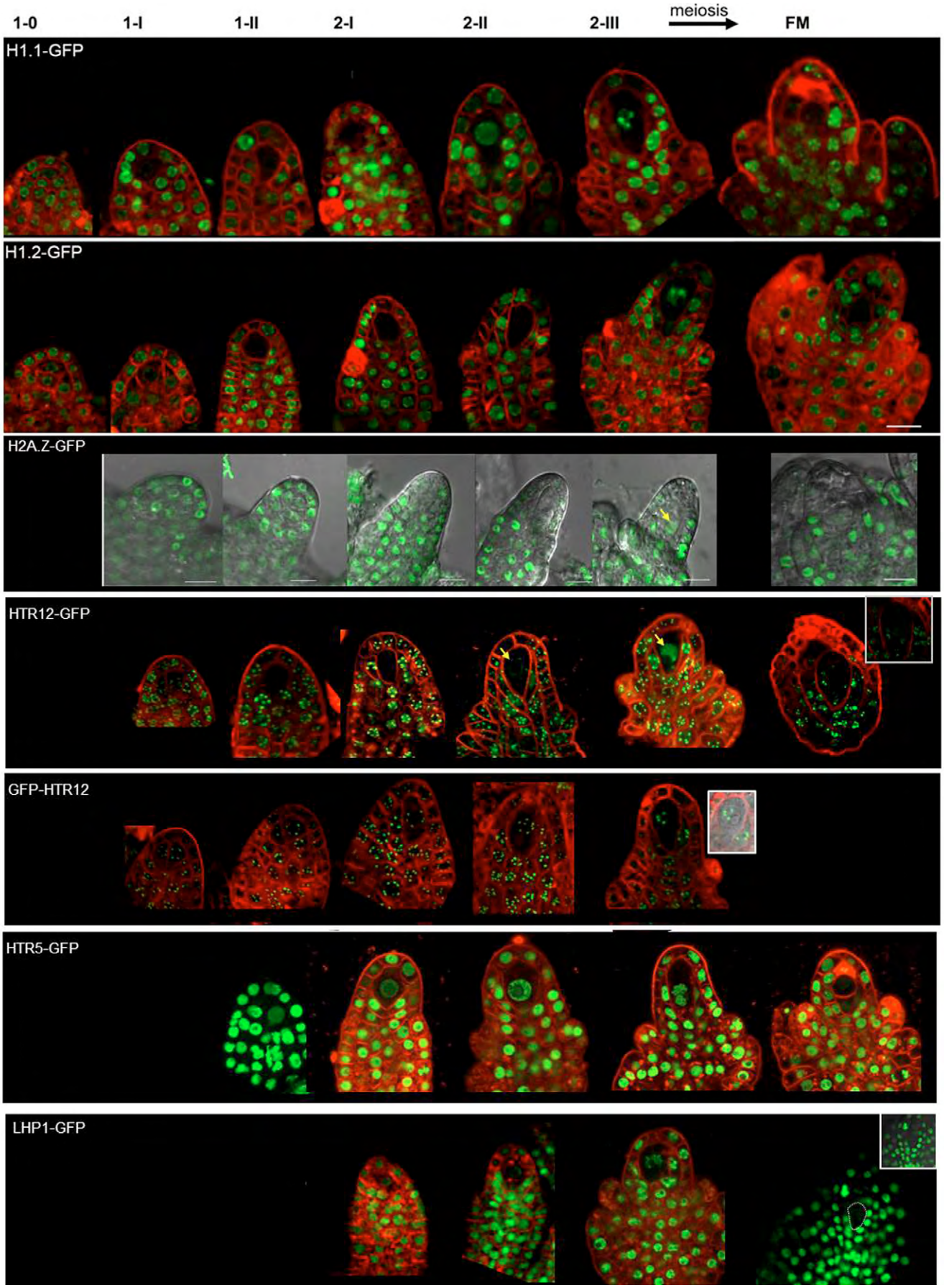


Fig. S1. Developmental dynamics of GFP-tagged histone variants and LHP1 during MMC differentiation. The expression dynamics of GFP-tagged histone variants is shown through the stages of ovule primordia development 1-0 to 2-III (onset prophase I) and in the functional megaspore.

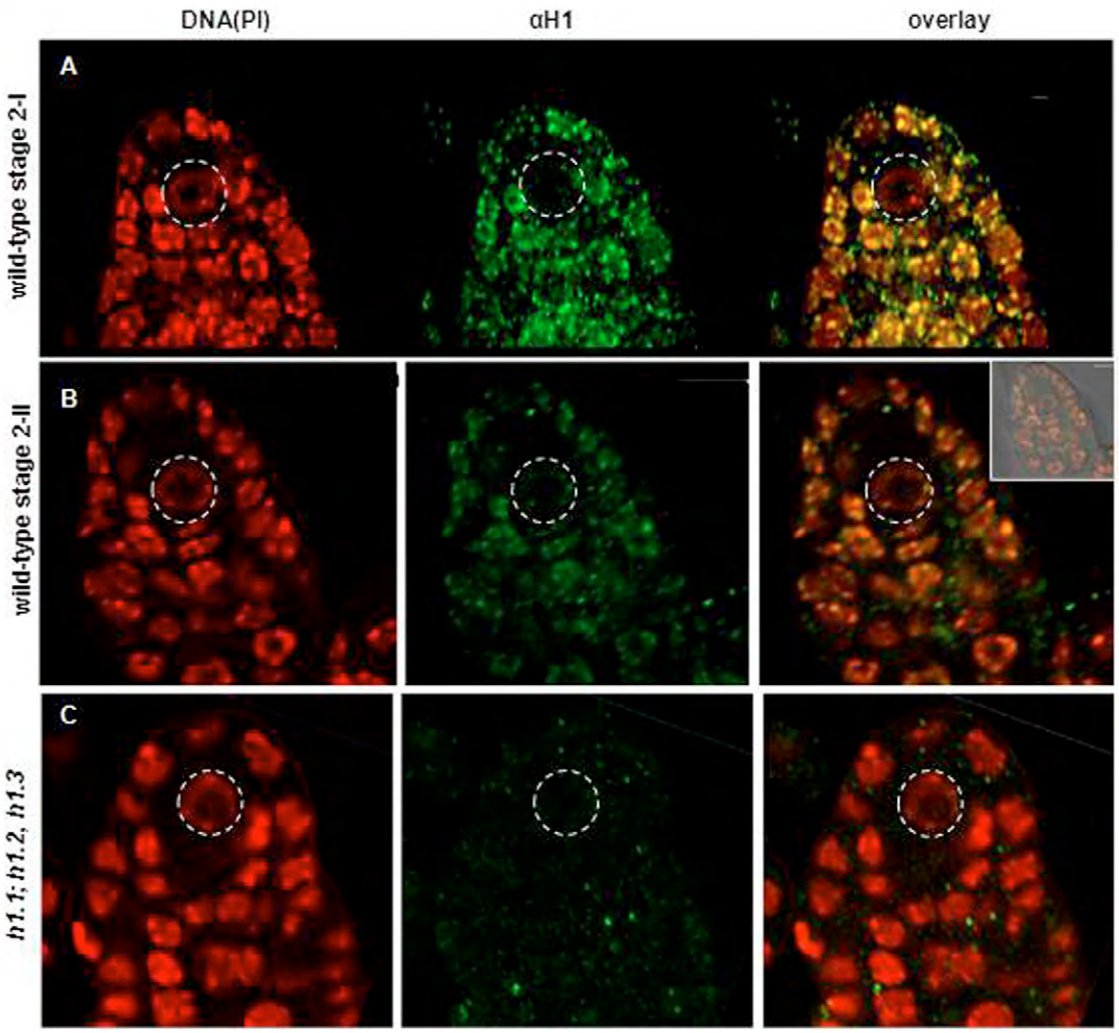


Fig. S2. H1 immunostaining with a novel, plant-specific antibody confirms H1 depletion in MMCs. (**A**) H1 is depleted in the MMC (dashed line) at stage 2-I, as revealed by whole-mount immunostaining in wild-type ovule primordia. (**B**) Reloading of H1 in the MMC at stage 2-II in wild-type ovule primordia (faint signals). (**C**) H1 is undetectable in the ovule primordia of *h1.1; h1.2, h1.3* triple mutant. The green signals arise from non-specific secondary antibody binding.

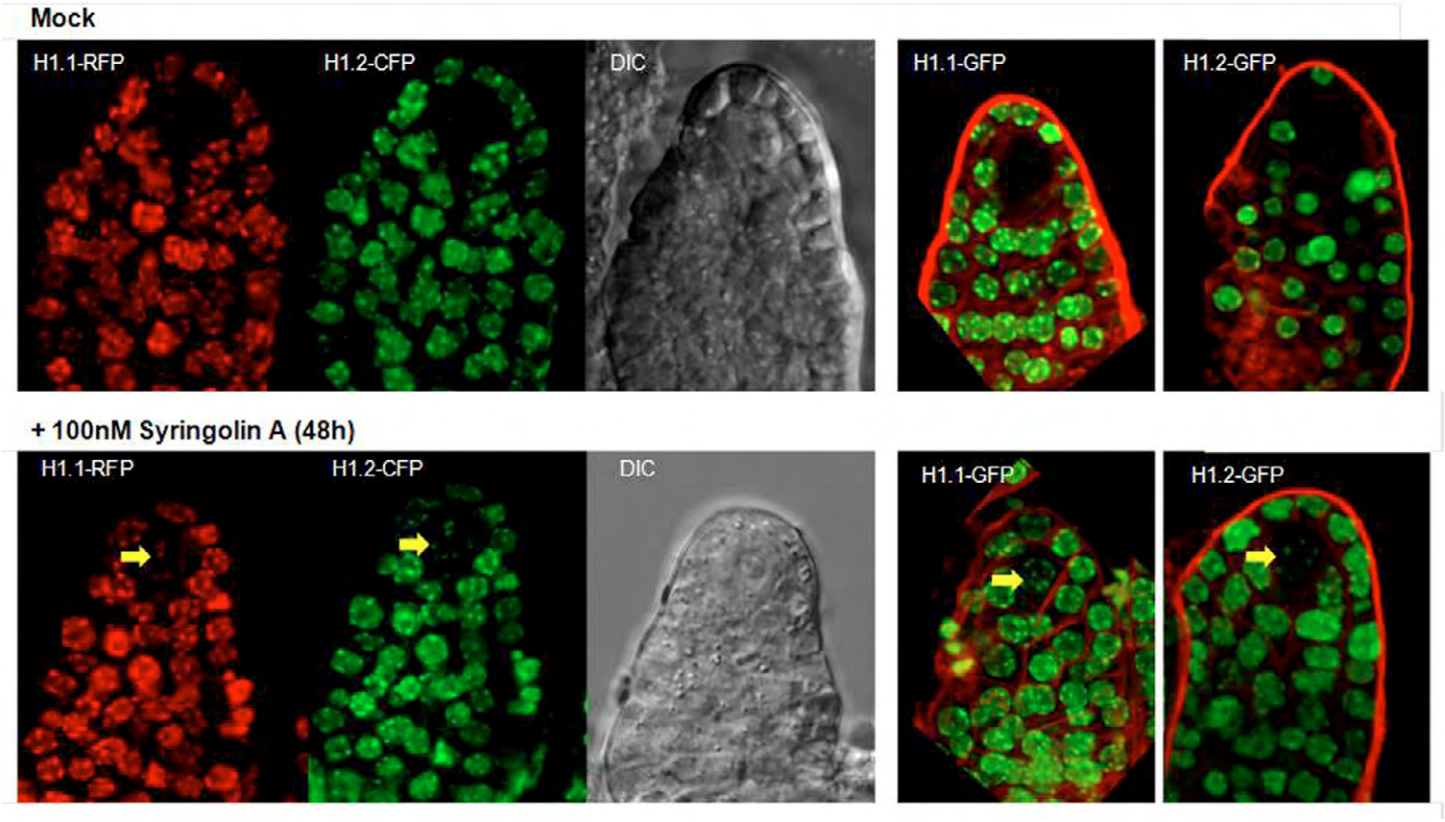


Fig. S3. GFP-tagged H1 dynamics is influenced by an inhibitor of the proteasome. Whole inflorescences were incubated in water (mock) or water containing 100 nM Syringolin A (Groll et al., 2008) for 48 hours before imaging (green, GFP; red, FM4-64; gray, DIC). Yellow arrows point to the MMCs where H1 signals clearly remain detectable.

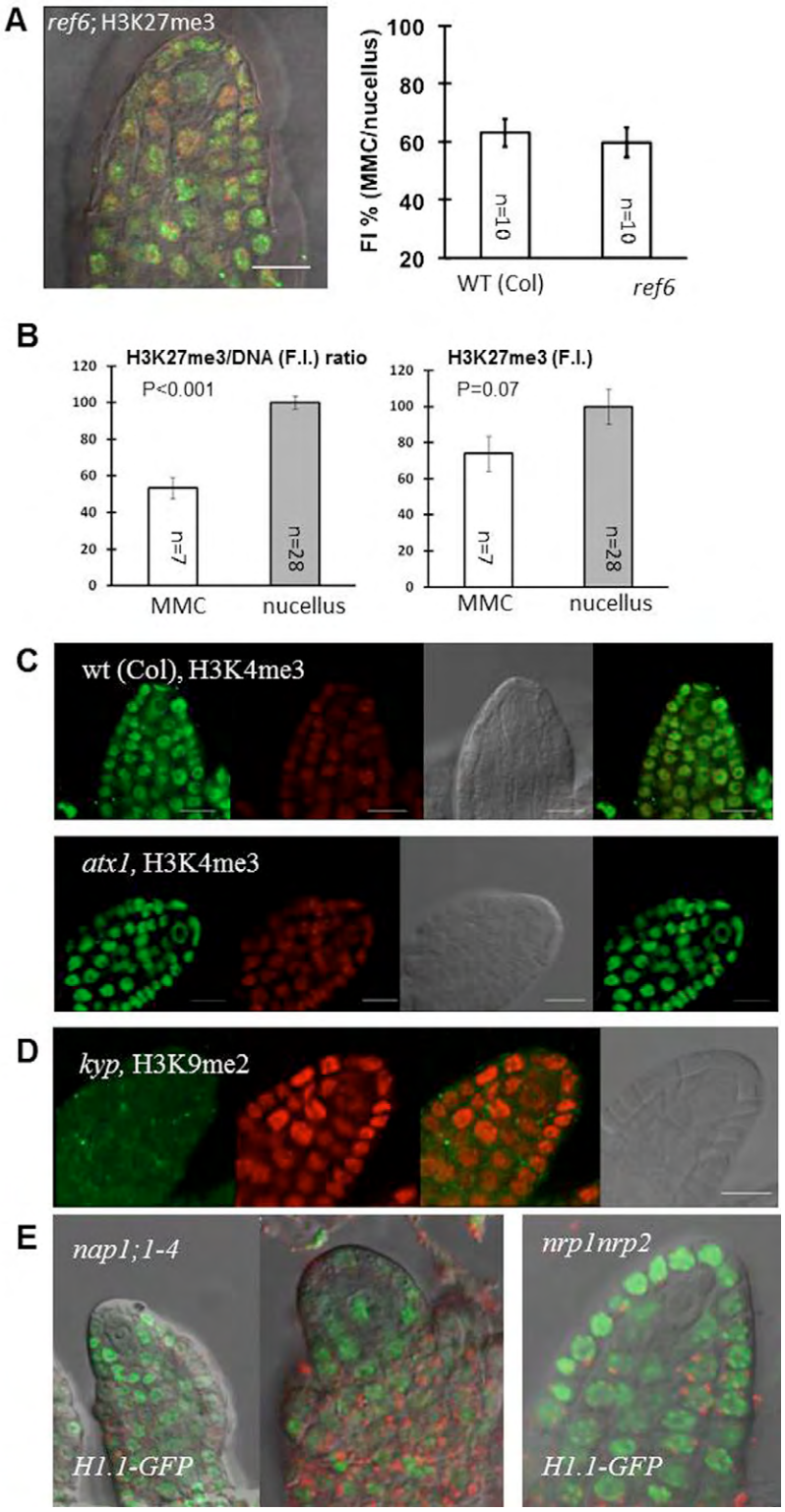


Fig. S4. Selected candidate modifiers do not contribute to chromatin dynamics in the MMC. (**A**) H3K27me3 levels in the MMCs relative to the nucellus are stable in the *ref6* mutant (Lu et al., 2011), compared with that in wild type. Representative image is shown for the overlay of antibody (green), DNA (propidium iodide, red) and transmitted light (DIC, gray). Detailed quantification is provided in Table S6. (**B**) Decreased levels of H3K27me3 in wild-type MMCs relative to the nucellus is visible both after normalization against the DNA content (left graph as in Fig. 3) or in absolute values of immunostaining signals (right graph), suggesting both a replication-coupled passive dilution and a probable active demethylation process (**C**) H3K4me3 levels in MMCs that lack ATX1 activity [*atx1-1* (Alvarez-Venegas et al., 2003)] are not increased, compared with that in *col*. Representative images show the antibody (green), DNA (propidium iodide, red), transmitted light (DIC, gray), and overlay of antibody (green) with DNA (red). (**D**) H3K9me2 immunosignals are drastically decreased in *kyp-2* (Lindroth et al., 2004) ovule primordia both in the MMC and nucellus, yet MMC and gametophyte differentiation proceeds normally. (**E**) H1.1-GFP is normally depleted in mutant MMCs of the quadruple mutant lacking NAP1;1-4 activity (Liu et al., 2009) or the double mutant lacking NRP1;NRP2 activity (Zhu et al., 2006) and reloaded at prophase I. Scale bars: 10 µm.

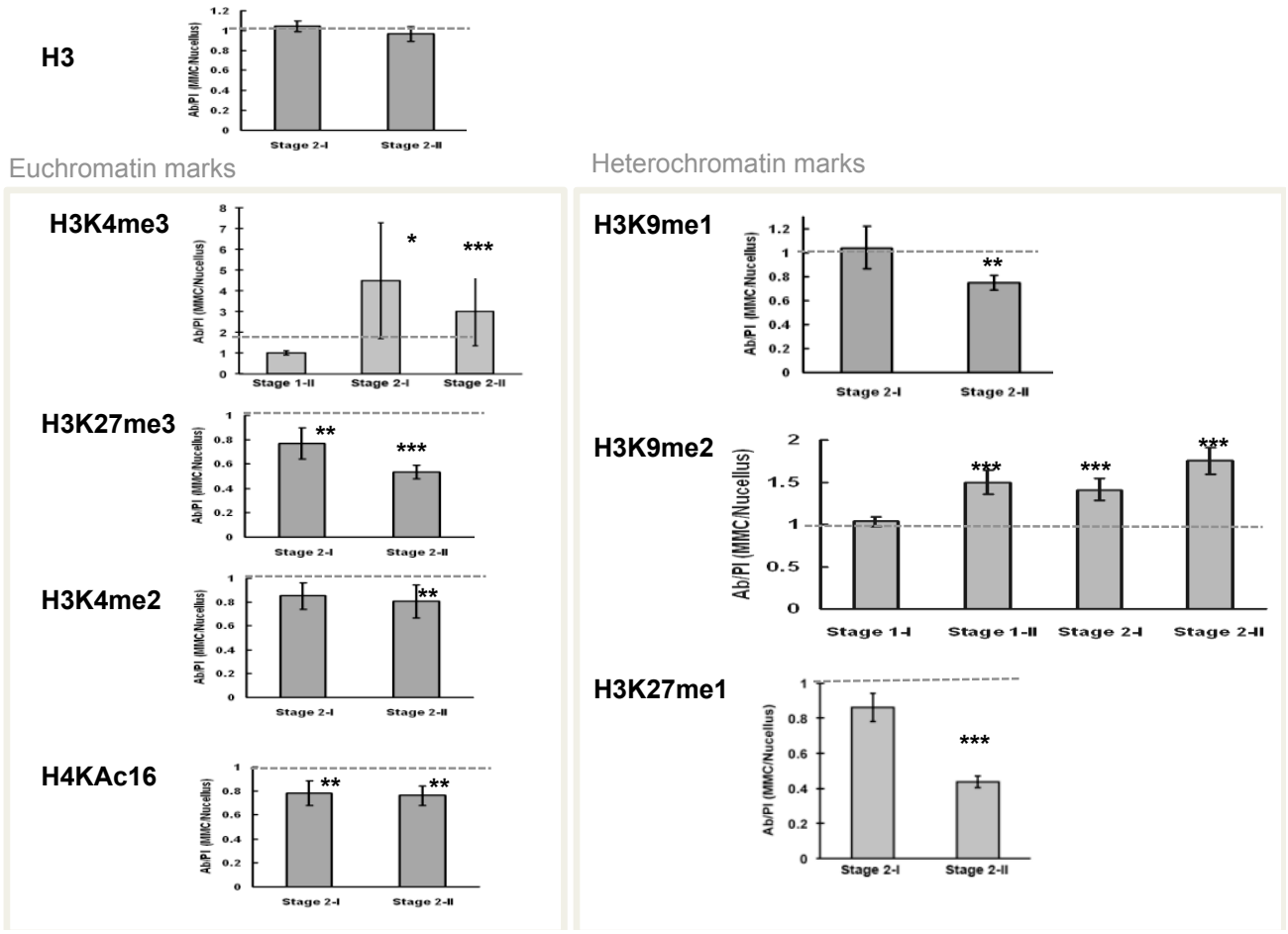


Fig. S5. Developmental dynamics of changes in chromatin modification levels in MMCs relative to nucellus cells. Graphs show the MMC/nucellus ratio of relative chromatin modification levels (measured as antibody signal intensity/DNA signal intensity). The stars indicate the level of significance in a Welch's *t*-test performed as for Fig. 3 (MMC versus nucellus). Most euchromatin marks are significantly altered in the MMC only at stage 2-I or 2-II, while H3K9me2 enrichment is typically measured at stage 1-II.

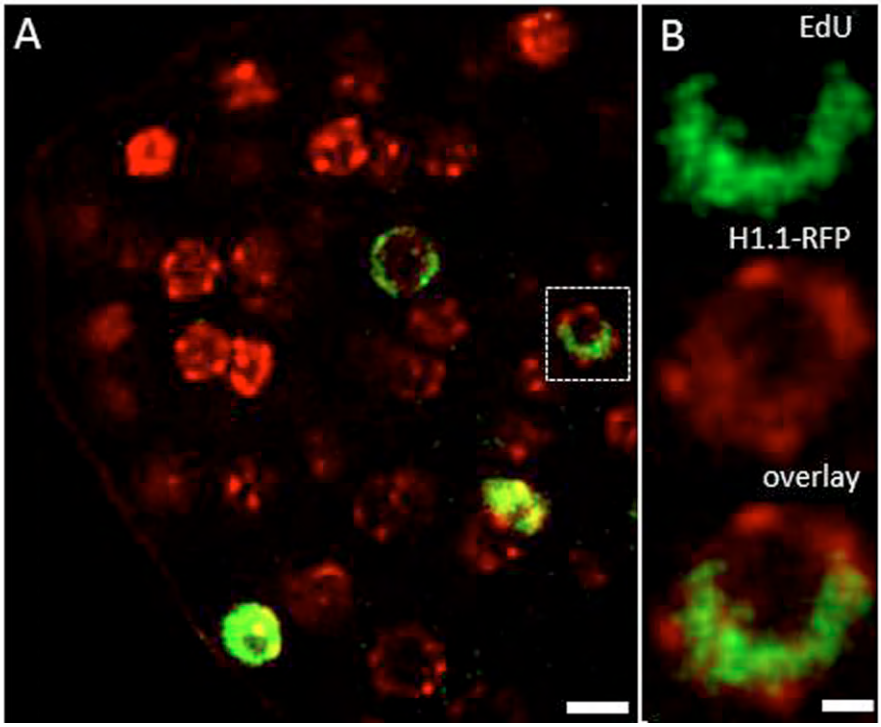


Fig. S6. H1.1 is a stable chromatin component during mitotic S phase. In mitotically active zones of the seedling root, H1.1::RFP (red) remains incorporated in the chromatin of nuclei engaged in S phase as revealed by EdU incorporation (green). (**A**) Confocal section of a root tip. (**B**) High magnification of one S-phase nucleus with EdU incorporation in euchromatin. After a 2-hour pulse, nuclei showed EdU incorporation in euchromatin only, heterochromatin only, or both. 21/21 nuclei with EdU incorporation in euchromatin only, as in B, showed H1.1-RFP signals. Scale bars: 10 µm.

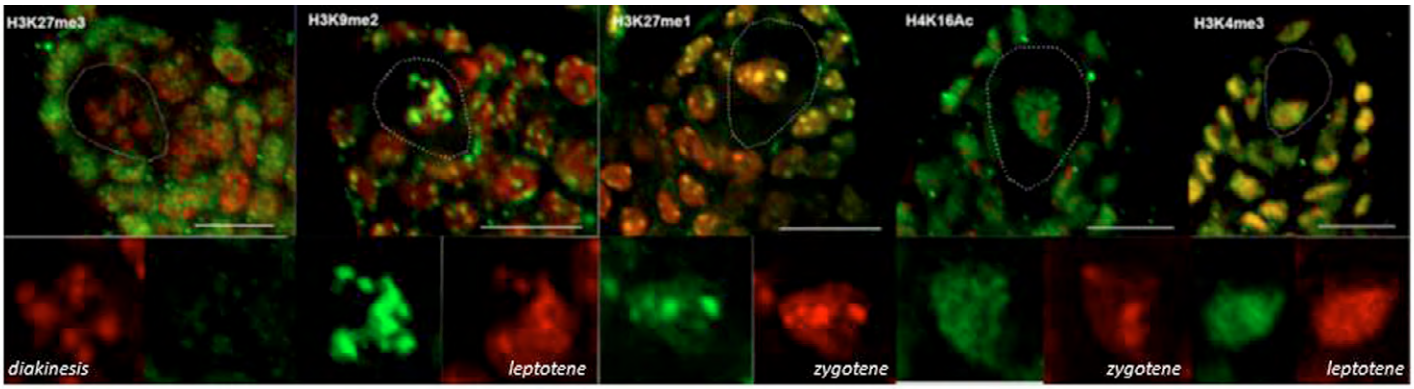


Fig. S7. Meiosis entails a novel dynamics in histone modifications. During meiosis I, the dynamic trends for H3K27me3 and H3K9me2 seen during MMC differentiation become more pronounced with a near loss and a high enrichment of immunosignals, respectively, while the trend appears to reverse for H3K27me1, with apparent higher signals than in differentiated MMCs (Fig. 3). H3K4me3 and H4K16Ac are well detected, yet the rapid evolution of meiotic chromosomes does not allow precise quantifications for these marks. The upper panel shows immunostaining signals of the indicated histone modifications in primordia tips at stage 2-III (dotted contours: MMC in prophase I). Beneath is shown a high magnification of MMC nuclei. Red, propidium iodide; green, antibody signal. Scale bars: 10 µm.

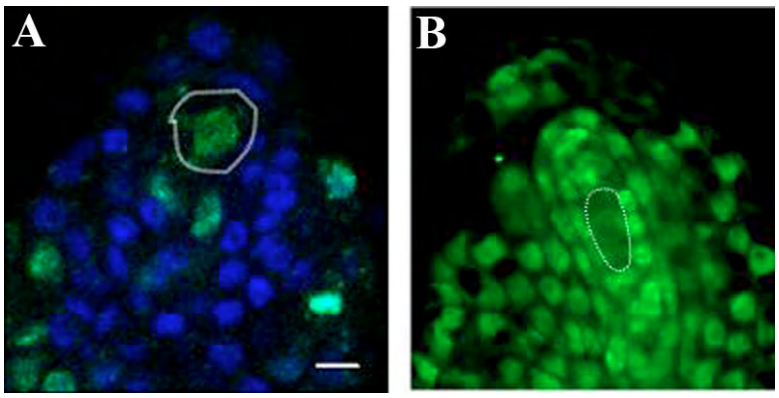
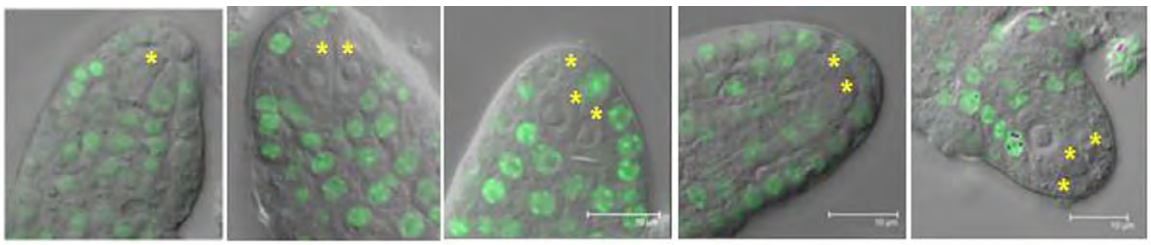


Fig. S8. Replication and transcriptional status in the FMS. (**A**) The selected product of female meiosis, the functional megaspore, rapidly engages in mitosis for gametophyte development, with EdU incorporation. (**B**) Active PolII is immunodetected at low and variable levels, probably due to the rapid transition into the gametophyte stage.



	Wild-type	ago9-3/+	ago9-4/+	rdr6-11/+	sgs3-11/+
Occurence phenotype	~ 5%	26%	31.5%	30.3 %	26.3%
Class A	5%	-	-	-	-
Class B	-	26%	21%	15%	23.6%
Class C	-	-	11.5%	15%	2.7%
n	100	30	57	33	38

Fig. S9. Loss of H1.1/GFP is a hallmark of multiple MMCs in *ago9*, *rdr6* and *sgs3* mutants. The number of primordia showing more than one enlarged cell (MMC and MMC-like) was scored in wild-type or mutant primordia of the indicated genotypes. The expression pattern of H1.1-GFP in these MMCs and MMC-like cells was scored: class A, primordia with only one H1.1-GFP-negative cell (MMC); class B, primordia where all MMCs are H1.1-GFP negative; class C, primordia with one H1.1-GFP-negative MMC and reduced H1.1-GFP levels in the other(s).

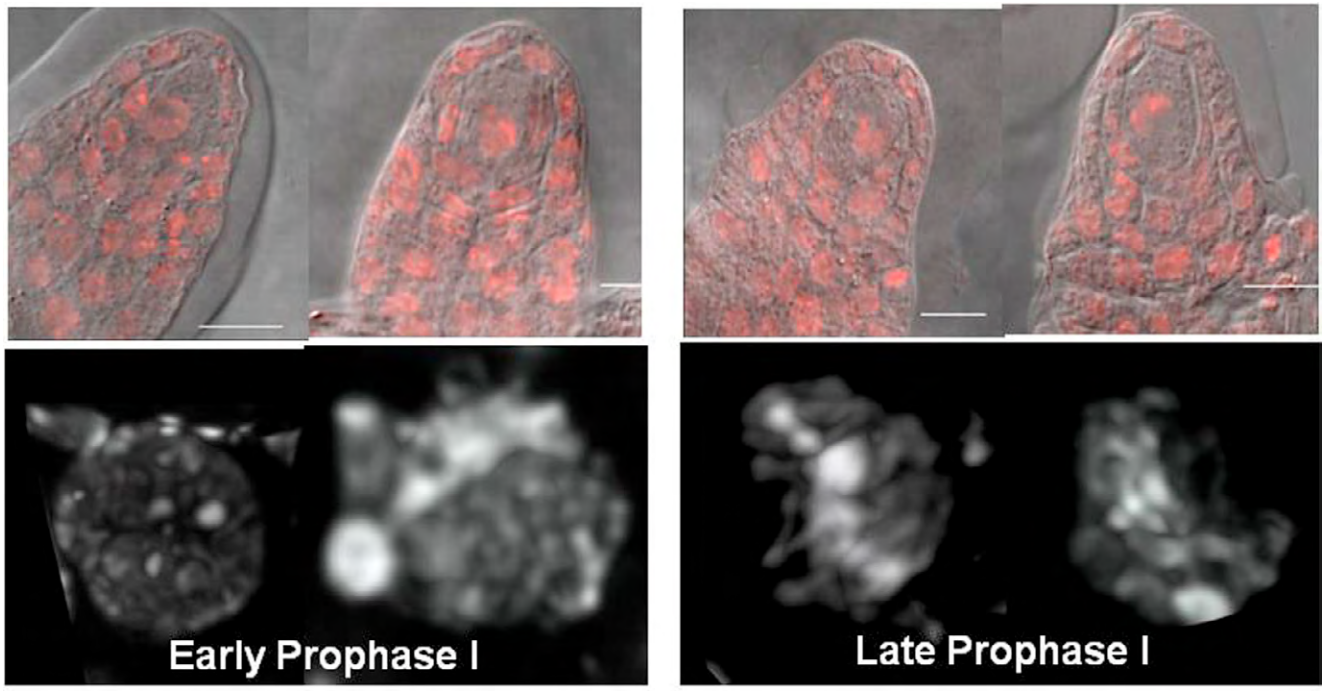


Fig. S10. Female meiosis in *sdg2*. Ovule primordia lacking SDG2 activity (Berr et al., 2010) undergo proper meiosis as assessed by clearing and DNA staining and despite reduced H3K4me3 levels compared with that in wild type (see also Fig. 6). The lower panel shows a deconvolved 3D reconstruction of MMC nuclei at different stages of prophase I corresponding to the primordia in the upper panel. Chromosome condensation is initiated during early prophase I (pre-leptotene/leptotene), and becomes more visible at late prophase, during which bivalents are formed. Images in the lower panel were deconvolved using Huygens (SVI).

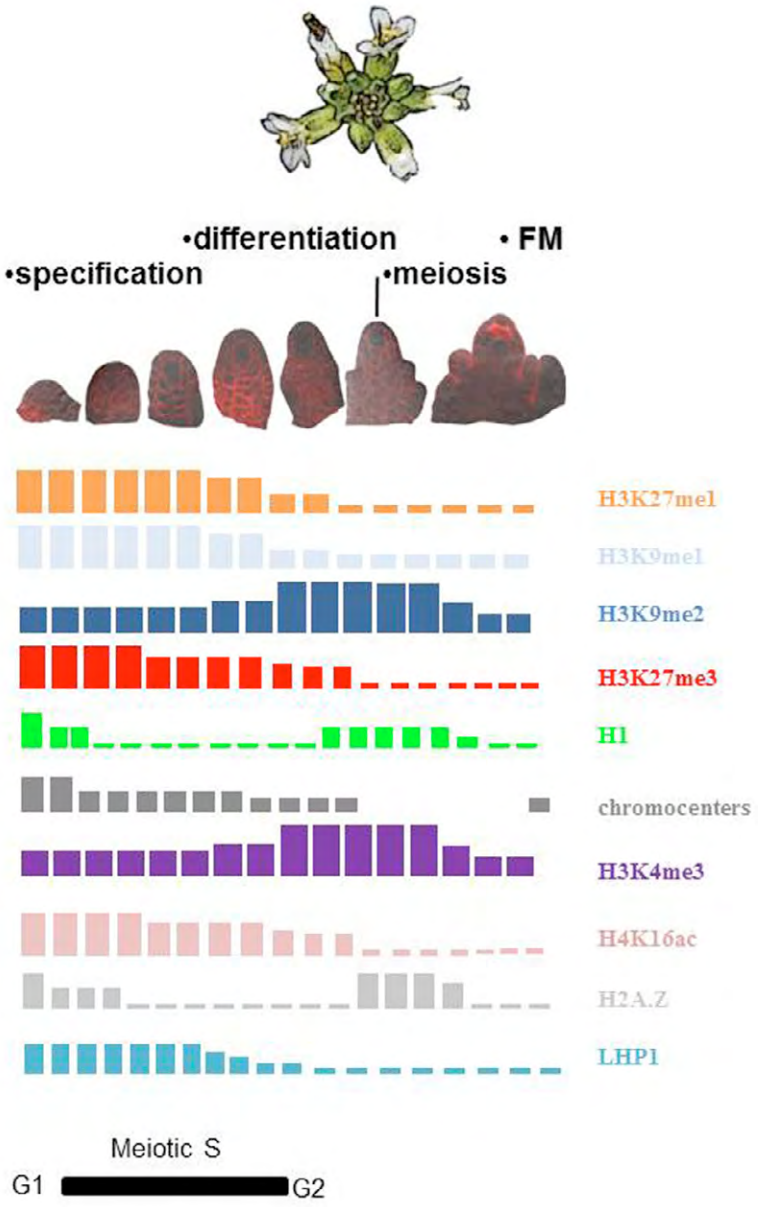


Fig. S11. Chromatin reprogramming in plant MMCs. Schematic representation of the chromatin changes occurring during MMC development and FM selection. Relative levels of chromatin modifications, chromatin components and heterochromatin in the MMC/ FM in comparison to nucellus cells are schematically represented by colored bars. The meiotic S phase concomitant to chromatin reprogramming is depicted in black. 'meiosis' on the top panel marks Prophase I, the following stages of meiotic division I and II are not shown.

Table S1. Detailed quantification of nuclear size and heterochromatin content in MMC and nucellus cells during ovule primordia development

Nuclear size (μm^3)	**MMC**			**nucellus**			
Stage	**average**	**s.d.**	***n***	**average**	**s.d.**	***n***	
1-II	44.2	± 16.8	(*n*=10)	36.698	± 11.46	(*n*=46)	*P*=0.206
2-I	76.127	± 33.66	(*n*=13)	43.072	± 15.4	(*n*=63)	*P*=0.0042
2-II	90.869	± 25.2	(*n*=10)	47.382	± 13.92	(*n*=50)	*P*=0.0003
RHF	**MMC**			**nucellus**			
Stage	**average**	**s.d.**	***n***	**average**	**s.d.**	***n***	
1-I	24.87	± 6.06	(*n*=10)	31.517	± 3.58	(*n*=30)	*P*=0.007
1-II	21.24	± 6.08	(*n*=10)	31.85	± 4.5	(*n*=30)	*P*=0.0002
2-I	18.38	± 7.84	(*n*=10)	31.38	± 4.44	(*n*=30)	*P*=0.0004
2-II	10.51	± 4.4	(*n*=10)	32.3	± 5.7	(*n*=30)	*P*<0.0001

Relative heterochromatin fraction (RHF) was calculated as a percentage ratio of fluorescence intensity (propidium iodide) in heterochromatic foci over intensity in the whole nucleus. s.d., standard deviation (note that the graphs show the standard error of the mean= s.d/$\sqrt{n}$). *P*-value is by Welch's *t*-test (two-tailed).

Table S2. Detailed quantifications of relative nuclear immunostaining in MMC and nucellus cells of primordia at stage 2-II

(A) Antibody signal over propidium iodide signal, relative to the nucellus

	MMC			nucellus			
	%	**s.d.**	***n***	**%**	**s.d.**	***n***	
H3	102.51	± 33.48	(*n*=9)	100	± 36.46	(*n*=43)	*P*=0.84
H3K9me1	73.54	± 14.23	(*n*=11)	100	± 20.74	(*n*=51)	*P*=3.57E–06
H3K9me2	171.44	± 47.72	(*n*=11)	100	± 31.21	(*n*=45)	*P*=1.69E–05
H3K27me1	43.57	± 8.25	(*n*=6)	100	± 20.23	(*n*=26)	*P*=6.70E–12
H3K27me3	53.51	± 15.16	(*n*=7)	100	± 19.02	(*n*=28)	*P*=7.58E–08
H3K4me2	74.56	± 17.28	(*n*=19)	100	± 28.88	(*n*=88)	*P*=1.74E–06
H3K4me3	270.17	± 263.77	(*n*=12)	100	± 24.30	(*n*=60)	*P*=0.03
H4KAc16	74.18	± 10.63	(*n*=10)	100	± 17.30	(*n*=46)	*P*=1.10E–07
RNA PolII	61	± 23.00	(*n*=7)	100	± 19.00	(*n*=25)	*P*=2.81E–04

(B) Antibody signal over propidium iodide signal, absolute ratios in the nucellus

	nucellus		
	Ab/PI	**s.d.**	***n***
H3	0.98664	± 0.36	(*n*=43)
H3K9me1	0.72185	± 0.15	(*n*=51)
H3K9me2	0.53354	± 0.17	(*n*=45)
H3K27me1	0.91792	± 0.19	(*n*=26)
H3K27me3	0.83598	± 0.16	(*n*=28)
H3K4me2	0.75001	± 0.22	(*n*=88)
H3K4me3	0.71775	± 0.17	(*n*=60)
H4KAc16	0.81903	± 0.14	(*n*=46)

The relative immunostaining signals are calculated as fluorescence intensity ratios of antibody (Ab) signals over propidium iodide (PI) signals. (A) The ratios in nucellus cells are averaged across *n* samples and set as 100%. Ab/PI ratio in the MMC relative to that in nucellus cells. (B) absolute ratios in nucellus cells. s.d., standard deviation (note that the graphs show the standard error of the mean=s.d/√*n*). *P*-value is by Welch's *t*-test (two-tailed).

Table S3. Detailed quantifications of relative nuclear immunostaining in MMC and nucellus cells of primordia at stage 1-II and 2-I

(A) Stage 1-II

(a) Antibody signal over propidium iodide signal, relative to the nucellus

	MMC			nucellus			
	%	**s.d.**	***n***	**%**	**s.d.**	***n***	
H3K9me2	150	± 36.14	(*n*=8)	100	± 22.43	(*n*=35)	*P*=0.0056

(b) Antibody signal over propidium iodide signal, absolute ratios in the nucellus

	Ab/PI	**s.d.**	***n***
H3K9me2	0.58919	± 0.59	(*n*=35)

(B) Stage 2-I

(a) Antibody signal over propidium iodide signal, relative to the nucellus

	MMC			***n*ucellus**			
	%	**s.d.**	***n***	**%**	**s.d.**	***n***	
H3	95.45	± 13.17	(*n*=10)	100	± 19.71	(*n*=48)	*P*=0.371
H3K9me1	99.37	± 31.34	(*n*=10)	100	± 71.85	(*n*=48)	*P*=0.965
H3K9me2	138.91	± 30.08	(*n*=10)	100	± 23.65	(*n*=44)	*P*=0.00035
H3K27me3	85.56	± 20.26	(*n*=9)	100	± 21.12	(*n*=37)	*P*=0.064
H3K4me2	86.49	± 32.27	(*n*=9)	100	± 25.30	(*n*=37)	*P*=0.248
H3K4me3	338.32	± 364.38	(*n*=6)	100	± 32.22	(*n*=29)	*P*=0.119
H4KAc16	74.34	± 17.87	(*n*=12)	100	± 30.25	(*n*=58)	*P*=0.000194

(b) Antibody signal over propidium iodide signal, absolute ratios in the nucellus

	Ab/PI	**s.d.**	***n***
H3	0.621242	± 0.06	(*n*=34)
H3K9me1	0.49682	± 0.18	(*n*=48)
H3K9me2	0.555674	± 0.07	(*n*=44)
H3K27me3	0.928	± 0.10	(*n*=37)
H3K4me2	0.640063	± 0.08	(*n*=37)
H3K4me3	0.666477	± 0.11	(*n*=29)
H4KAc16	0.802813	± 0.12	(*n*=58)

See Table S2 for legend.

Table S4. Quantification of DNA content increase and EdU incorporation in MMCs

(A) Quantification of DNA content (PI fluorescence intensity) in MMCs relative to the averaged content in L1-1, L1-0 and L1-2 nucellus cells during ovule primordia development

stage	average	s.d.	*n*
1-I	1.01	± 0.12	10
1-II	1.24	± 0.32	10
2-I	1.59	± 0.37	10
2-II	1.96	± 0.40	10

s.d., standard deviation (note that the graphs show the standard error of the mean= s.d./$\sqrt{n}$).

(B) Quantification of MMCs with distinct EdU incorporation patterns (2-hour pulse)

EdU signal	Stages 1-I + 1-II	Stage 2-II
Euchromatin only (1)	1	16
Heterochromatin only (2)	10	1
Euchromatin and heterochromatin	1	–
Total	**12**	**17**

Table S5. Detailed quantification of relative nuclear immunostaining in functional megaspore and nucellus cells

(A) Antibody signal over propidium iodide signal, relative to the nucellus

	MMC			nucellus			
	%	**s.d.**	***n***	**%**	**s.d.**	***n***	
H3	101.24	± 17.40	(*n*=6)	100	± 20.18	(*n*=24)	*P*=0.881
H3K9me1	77.72	± 29.65	(*n*=10)	100	± 45.06	(*n*=50)	*P*=0.054
H3K9me2	76.13	± 17.86	(*n*=6)	100	± 28.69	(*n*=25)	*P*=0.015
H3K27me1	66.96	± 15.59	(*n*=7)	100	± 25.21	(*n*=32)	*P*=7.12E-05
H3K4me2	50.34	± 18.05	(*n*=11)	100	± 20.62	(*n*=54)	*P*=2.29E-11
H3K4me3	64.74	± 10.40	(*n*=12)	100	± 15.19	(*n*=51)	*P*=8.84E-14
H4KAc16	56.96	± 13.66	(*n*=12)	100	± 35.57	(*n*=58)	*P*=1.19E-09

(B) Antibody signal over propidium iodide signal, absolute ratios in the nucellus

	Ab/PI	**s.d.**	***n***
H3	0.699	± 0.14	(*n*=24)
H3K9me1	0.673	± 0.30	(*n*=50)
H3K9me2	0.663	± 0.19	(*n*=24)
H3K27me1	0.982	± 0.25	(*n*=32)
H3K4me2	1.157	± 0.24	(*n*=54)
H3K4me3	0.920	± 0.14	(*n*=51)
H4KAc16	1.330	± 0.47	(*n*=58)

s.d., standard deviation (note that the graphs show the standard error of the mean=s.d./√*n*).

Table S6. Detailed quantifications of relative nuclear immunostaining in *ago9-4* and *sdg2* mutant megaspore mother cells relative to nucellus cells

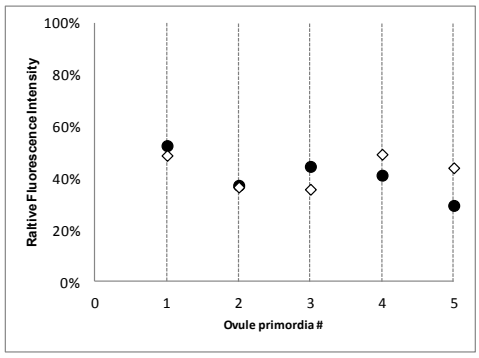
Ab/Pi, fluorescence intensity sum of the antibody (Ab) relative to the DNA (PI) signals. s.d., standard deviation (note that the graphs show the standard error of the mean=s.d/√*n*). *n*, number of cells quantified. *P*-value by Welch's *t*-test (two-tailed).

(**A**) **H3K27me1 relative levels in *ago9-4* MMCs**

(a) Antibody signal over propidium iodide signal, relative to the nucellus in *ago9-4/ago9-4* mutant

	Ab/PI	**s.d.**	***n***	
mmc1	38.43	±11.61	(*n*=5)	*P*=0.7
mmc2	39.65	±8.31	(*n*=5)	
nucellus	100	±30.24	(*n*=24)	

In each primordia, the mmc with the highest intensity was called mmc1, whereas that with the lowest was called mmc2. The distribution of fluorescence intensity in individual mmcs (white and black dot) per ovule primordia is as follows:



(b) Antibody signal over propidium iodide signal, absolute ratios in the nucellus in *ago9-4/ago9-4* mutant

	Ab/PI	**s.d.**	***n***
nucellus	0.997043	± 0.3016	(*n*=24)

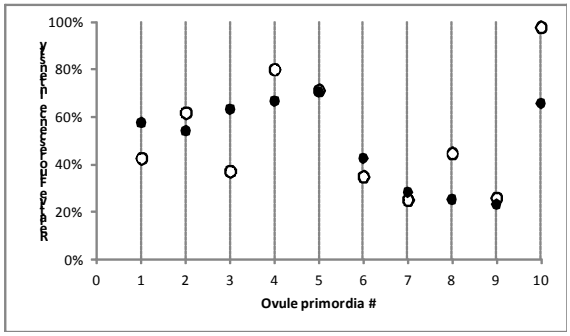
(**B**) **H3K27me3 relative levels in *ago9-4* MMCs**

(a) Antibody signal over propidium iodide signal, relative to the nucellus in *ago9-4/ago9-4* mutant

	Ab/PI	**s.d.**	***n***	
mmc1	62.82	±38.01	(*n*=10)	*P*=0.38
mmc2	48.63	±32.7	(*n*=10)	
nucellus	100	±48.25	(*n*=50)	

In each primordia, the mmc with the highest intensity was called mmc1, whereas that with the lowest was called mmc2. The distribution of fluorescence intensity in individual mmcs (white and black

dot) per ovule primordia is as follows:



(b) Antibody signal over propidium iodide signal, absolute ratios in the nucellus of the *ago9-4/ago9-4* mutant

	Ab/PI	**s.d.**	***n***
nucellus	1.9505	± 0.89	(*n*= 50)

(C) H3K4me3 relative levels in *sdg2* versus wild-type (Col) MMCs

(a) Antibody signal over propidium iodide signal, relative to the nucellus in *sdg2/sdg2* mutant

MMC			**nucellus**			
%	**s.d.**	***n***	**%**	**s.d.**	***n***	
163.29	± 48.59	(*n*=12)	100	± 54.16	(*n*=59)	*P*=0.0001

(b) Antibody signal over propidium iodide signal, absolute ratios in the nucellus of the *sdg2/sdg2* mutant

Ab/PI	**s.d.**	***n***
1.26	± 0.68	(*n*=59)

(c) Comparison of H3K4me3 relative levels (MMC/nucellus) in *sdg2* and wild-type (Col) primordia

	average	**s.d.**	***n***	
Col	270.17	131.89	12	*P*=0.0152
sdg2	163.29	48.59	12	

(D) H3K27me3 relative levels in *ref6* versus wild-type (Col) MMCs

(a) Antibody signal over propidium iodide signal, relative to the nucellus of the *ref6/ref6* mutant

MMC			**nucellus**			
%	**s.d.**	***n***	**%**	**s.d.**	***n***	
59.77	± 16.3	(*n*=10)	100	± 17.15	(*n*=50)	*P*=0.004

(b) Antibody signal over propidium iodide signal, absolute ratios in the nucellus of the *ref6/ref6* mutant

Ab/PI	**s.d.**	***n***
0.79	± 0.14	(*n*=50)

(c) Comparison of H3K4me3 relative levels (MMC/nucellus) in *ref6* and wild-type (Col) primordia

	average	**s.d.**	***n***
Col	63.16	30.8	10
ref6	59.77	32.6	10

See Table S2 for legend.

Table S7. List of antibodies and test of immunostaining signal reliability in whole-mount ovule primordia in serial dilutions

				Dilution for immunostaining			
Antibody target	**Provider**	**Cat#**	mg/ml	**1/1000**	**1/500**	**1/200**	**1/100**
H3	Abcam	ab1791	1	-	-	+	nd
H3K27me1	Upstate	07-448	1	nd	-	+	nd
H3K27me3	Upstate		1	-	-	+	+
H3K4me2	Abcam	ab32356	0.04	-	-	+	nd
H3K4me3	Upstate	07-473	0.1-0.5	+	+	+	+
H3K9me1	Upstate	07-450	1	nd	-	+	nd
H3K9me2	Upstate	07-441	1	nd	-	+	nd
H4K16Ac	Millipore	07-329	1	-	-	+	nd
H1	Agrisera	AS111801	1	nd	nd	+	nd

Immunostaining results on whole-mount primordia: +, stable signal; -, unstable signals (not reproducible across replicates). Gray: dilutions used for the quantifications in Figs 3, 5, 6, S2 and Tables S2, S3, S5, S6. nd, not determined.

Table S8. Primers used for generating GFP-tagged variants

Vector	**promH1.1::H1.1-EGFP**
amplicon	H1.1 promoter + coding sequence
primers	5'-GCGTCGACTCATTCTGTGATAGGGATGG-3'
	5'-GCCCATGGGCTCTCCAAAGGTTAGTTTT-3'
cloning sites	*Sal*I, *Bam*HI
amplicon	H1.1 termination sequence
primers	5'-GCGAATTCTGAAGTTAGGGTTTGTAGGTAG-3'
	5'-GCCCATGGGCTCTCCAAAGGTTAGTTTT-3'
cloning sites	*Eco*RI, *Nco*I
vector	**promH1.1::EGFP-H1.1**
amplicon	H1.1 promoter
primers	5'-GCGTCGACTGTTGGGGAAGATAATCCAA-3'
	5'-GCGGATCCCATCGTCTTCTGAACTTAAGATC-3'
cloning sites	*Sal*I, *Bam*HI
amplicon	H1.1 coding sequence + termination sequence
primers	5'-GCGAATTCTCAGAGGTGGAAATAGAGAACG-3'
	5'-GCCCATGGTGGTAAGCCATCCACAAACA-3'
cloning sites	*Eco*RI, *Nco*I
vector	**promH1.1::RFP_T-H1.1**
amplicon	RFP_T
primers	5'-TTAGGATCCGTGTCTAAGGGCGAAGAGC-3'
	5'-ATTAGAATTCCTTGTACAGCTCGTCCATGCC-3'
cloning sites	*Bam*HI, *Eco*RI
vector	**promH1.2::H1.2-EGFP, promH1.2::H1.2-CFP**
amplicon	H1.2 promoter + coding sequence
primers	5'-GCCTGCAGTTCGTAAATGGTAGATGGAAAACA-3'
	5'-GCGTCGACCTTCTTAGCCTTCCTAGTCGAA-3'
cloning sites	*Pst*I, *Sal*I
amplicon	H1.2 termination sequence
primers	5'-GCGAATTCTGAAGAAGATTGGTTTAGGAT-3'
	5'-GCGCTAGCTTCGAGGAATTAGGTGAGAA-3'
cloning sites	*Eco*RI, *Nhe*I
vector	**promH1.2::EGFP-H1.2**
amplicon	H1.2 promoter
primers	5'-GCCTGCAGGCAGTTCGTAAATGGTAGATGG-3'
	5'-GCGTCGACCATCTTCTTCTCTCTCAGAAACTG-3'
cloning sites	*Pst*I, *Sal*I
amplicon	H1.2 coding sequence + termination sequence

primers	5’-GCCAATTGTCTATAGAGGAAGAAAACGTTCC-3’
	5’-GCGCTAGCTCACAAGAGGTTTGCGAATG-3’
cloning sites	*Mun*I, *Nhe*I

Supplementary data for *sdg2* mutant

Mutation of *sdg2* leads to ectopic MMCs formation, which impairs linker histone variant H1.1deposition

The mutation of *sdg2* in *Arabidopsis*, with reduced H3K4me3 levels in MMCs, allows for normal meiosis progression, but impairs postmeiotic gametophyte development, indicating that chromatin reprogramming possibly contributes to establishing competence for postmeiotic gametophyte development (Figure 1). Surprisingly, supernumerary MMCs were obseved in 36.3% of ovule primordia in *sdg2* homozygous mutant, although most of them fail to induce further gametophyte development (Berr et al., 2010, Figure 1G, Figure 2). These multiple MMCs may be generated by overproliferation of archespores due to compromised H3K4 trimethylation. However, the MMC specific marker *KNUCKLES* was not always expressed in all enlarged MMC-like cells, suggesting a *KNUCKLES*-independent pathway was likely to be involved in formation of these multiple MMCs (Figure 3). It is noteworty that reduction of H3K4me3 not only influences MMC fate, but nucellar cells where the linker histone variant H1.1 deposition was affected, with its eviction in *sdg2* mutant (Figure 4).

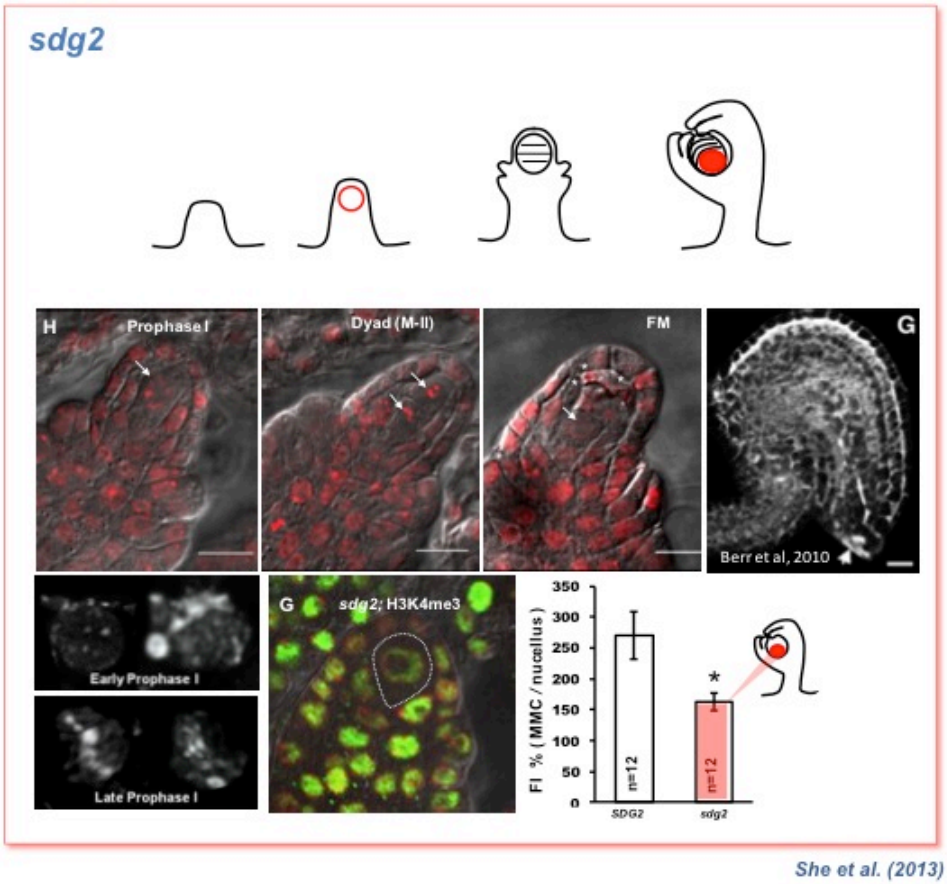


Figure 1. Chromatin dynamics, conferred by H3K4me3, is likely to contribute to establishing competence for postmeiotic gametophyte fate (She et al., 2013). The *sdg2* mutation in ovule primordia leads to reduced H3K4me3 in MMCs relative to nucellus (G), which doesn't affect meiotic progression (H), but impairs post-meiotic gametophyte development (G, Berr et al., 2010).

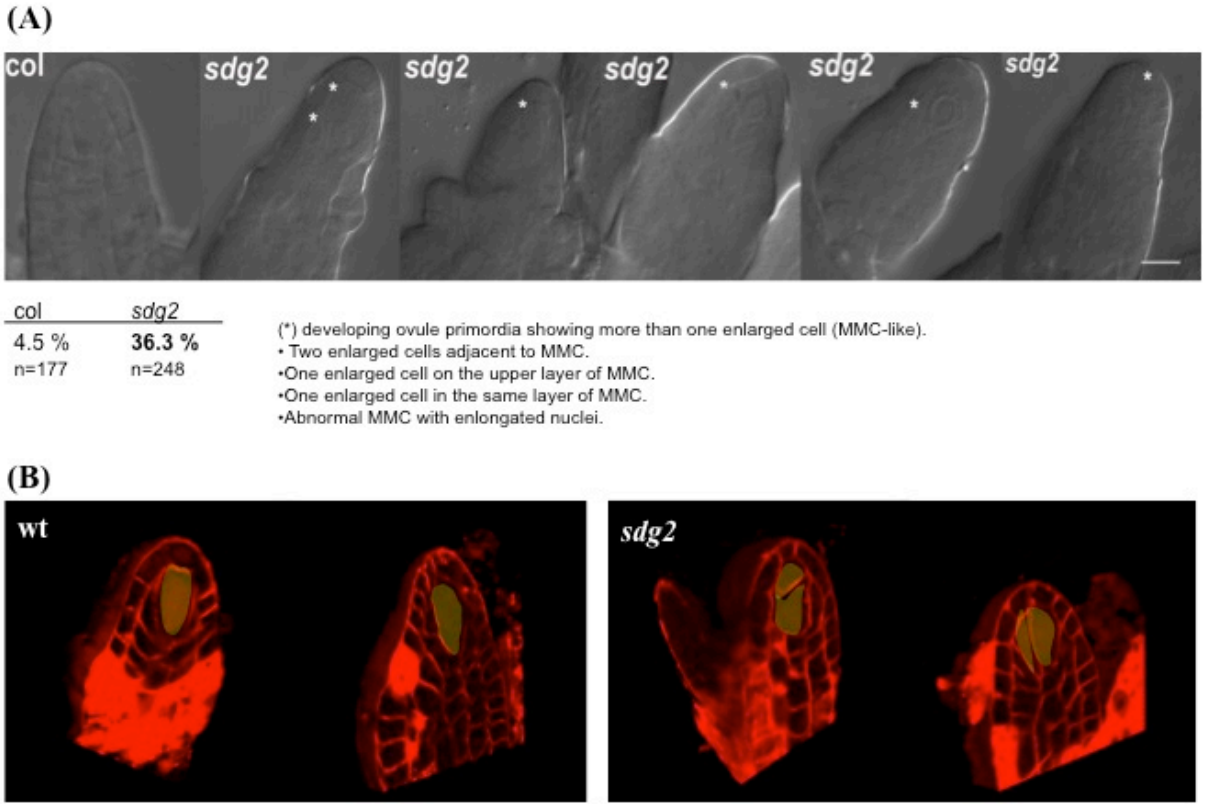


Figure 2. *SDG2* loss-of-function in the nucellus leads to ectopic MMCs. Multiple MMCs are formed in ovule primordia lacking *SDG2* activity, with a ratio of 36.3% compared to 4.5% in wild type, as observed by clearing (A, supernumerary MMCs were marked with asterisk in white) and cellular membrane staining (B, MMCs were illustrated in dark yellow, crosses were done by me and images were from C.Baroux).

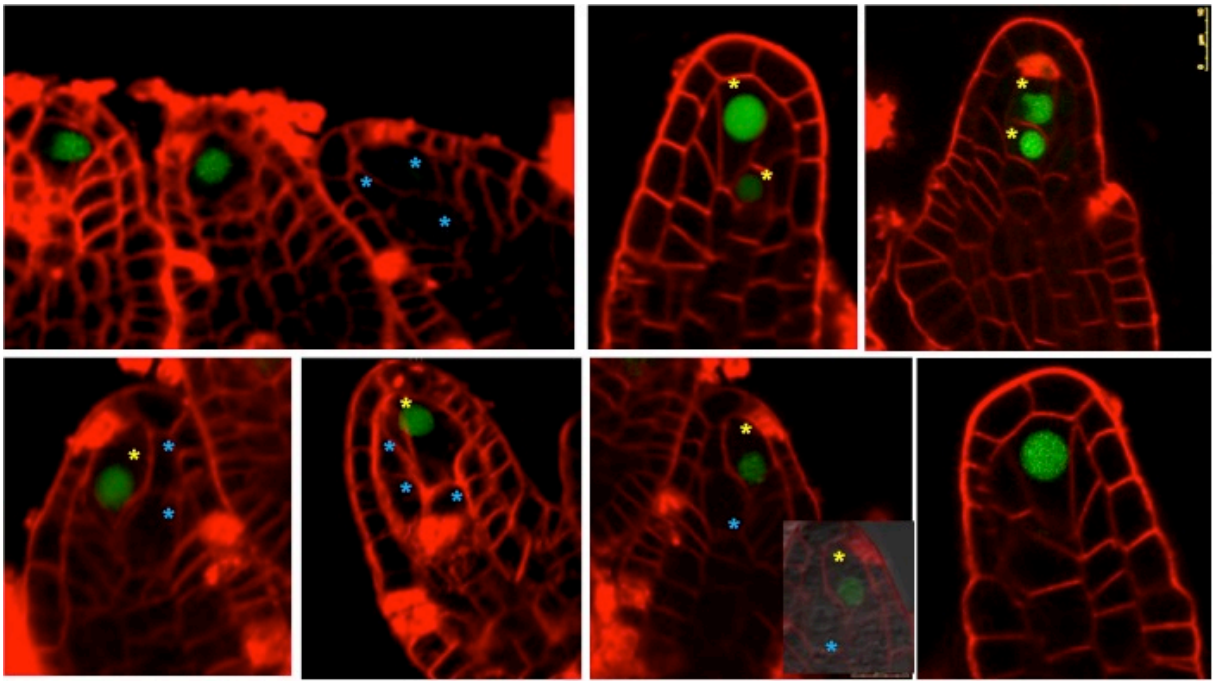


Figure 3. Ectopic MMCs in *sdg2* do not always express pKNU-nlsYFP. As observed from the *sdg2/sdg2*;pKNU-nlsYFP ovule primordia with the cellular membrane stained with FM®4-64, YFP fluorescence driven by *KNUCKLES* promoter is either absent from all MMCs, or only present in only 1-2 MMCs in *sdg2* ovule primordia. MMCs are illustrated with asterisk. Crosses were done by me. Images were from C.Baroux.

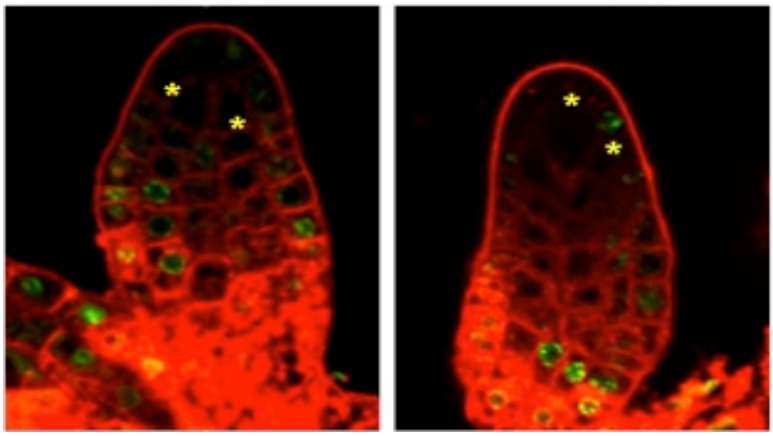


Figure 4. The *sdg2* mutation disables H1.1-GFP deposition. Linker histone variant H1.1 is not only evicted in ectopic MMCs, but also in the nucellus, as observed in *sdg2/sdg2*;pH1.1:H1.1-GFP line. MMCs are marked with yellow asterisk. Crosses were done by me and images were from C.Baroux.

5. Result Chapter III

Developing a strategy enabling epigenome profiling of MMCs

Abstract

In flowering plants, female megaspore mother cells (MMCs) differentiate late in ovule primordia within the floral gynoecium during sporogenesis, which marks the somatic-to-reproductive lineage fate transition. This process entails extensive chromatin reprogramming, characterized by chromatin decondensation, dynamic changes of chromatin composition and histone modifications, including a quantitative increase of a permissive mark H3K4me3 and a decrease of a repressive mark H3K27me3 in MMCs.

In order to identify the loci affected by these epigenetic changes, we tried to develop a strategy enabling comparative profiling of chromatin-immunoprecipitated fractions using antibodies against H3K27me3 and H3K4me3 modifications in the MMC and the nucellus (ChIP-seq). The first aim is to isolate MMCs and nucellus nuclei for further purification. While the next is to analyze the epigenome profile of chromatin specifically from the MMC and the nucellus using the cell-specific purification system (first aim) and ChIP-seq.

For this project, I was still at the stage of MMC nuclei isolation for profiling, with two approaches currently tested: INTACT system and Fluorescence assisted cell sorting (FACS) using pKNU-nlsYFP lines. The work for MMC epigenome profiling is summarized here.

Introduction

In flowering plants, the germinal lineage is established late during development (Baroux et al., 2011). It is initiated by the differentiation of spore mother cells (SMCs) in male and female flower organs, which marks the transition from a somatic to reproductive cell fate. The female megaspore mother cells (MMCs) differentiate in the nucellus of ovule primordia. Plant MMCs can be viewed as the functional equivalent of animal Primordial Germ Cells (PGCs) that will form the gametes. Epigenetic regulation plays fundamental role in diverse biological processes ranging from transcriptional regulation to global shaping of developmental landscapes (He et al., 2011). PGCs also undergo epigenetic reprogramming, characterized by

global DNA demethylation, alteration of histone modifications, X-chromosome reactivation and imprint erasure on embryonic day 8.5 (E8.5) and E11.5, which is required for removal of epigenetic barriers to totipotency (Hajkova et al., 2008; Hackett et al., 2012). In flowering plants, epigenetic reprogramming including dynamic patterns of DNA methyltransferase expression, DNA methylation, distribution of histone variants and histone modifications has been described after meiosis during gametophytic development (Ingouff et al., 2010; Houben et al., 2011; Calarco et al., 2012). Recently, we have also demonstrated that extensive epigenetic reprogramming takes place before meiosis using quantitative whole-mount immunostaining approach, which underlies acquisition of competence for postmeiotic, gametophyte fate (She et al., 2013). We found drastic nuclear reorganization and changes in chromatin composition in MMCs of *Arabidopsis*, including a reduction of the *Polycomb* silencing-associated mark H3K27me3 and increase of the *Trithorax* permissive transcription-associated mark H3K4me3 (She et al., 2013).

So far, the interplay between epigenetic modifications and transcriptional regulatory network, as well as the dynamics of epigenome during cell fate transition, remains poorly understood. Genome-wide chromatin profiling was performed in several plant tissues or plant cell cultures, highlighting the complexity of the epigenetic code and the combinatorial potential by chromatin indexing (Deal and Henikoff, 2010; He et al., 2012). Yet, the relevance of chromatin indexing in cell fate transition and cell differentiation can only be resolved by epigenome analysis at single-cell-resolution. This, however, is still hindered by the high cellular dilution of target cells within tissues (Wuest et al., 2012). The recently published INTACT system allows for chromatin profiling of individual cell types from *Arabidopsis* root epidermis, thereby circumventing the technical limitations for rare cell type isolation. In this system, a nuclear envelope protein in the target cell type is labeled with biotin and allows for affinity isolation (Deal and Henikoff, 2010).

To identify the differences of gene expression affected by those epigenetic changes in MMCs, we were trying to perform the comparative profiling of the chromatin from MMCs and surrounding nucellar cells using antibodies against H3K27me3 and H3K4me3. We tested two methods for MMC nuclei isolation: 1) INTACT system, 2) FACS using the mark line (pKNU-nlsYFP).

Materials and Methods

Plant Material and growth conditions

Arabidopsis plants were grown under long-day condition (16 hours light/8 hours dark) at 18-20°C in a plant growth chamber. pKNU-nlsYFP line for Fluorescence Assisted Cell Sorting (FACS) was from group Koltunow (Janousek et al., 2000; Tucker et al., 2012). The line pRPS5A:hBirA-HA was from Dr. O. Boyko (Grossniklaus lab). The seeds for all the lines used for this work were sterilized in 3% NaClO, with 0.01% Triton for 10min, and rinsed by ddH_2O for five times, then sterilized in 70% ethanol for 2 min, followed by 1× ddH_2O wash, and transferred into plates with MS medium (Murashige & Skoog germination medium with bactoagar), sealed with 3M tape. The plate with seeds was kept for 2 days at 4°C, and then transferred to growth cabinet.

Construction of vectors

The nuclear tagging fusion fragment (NTF) was amplified from GL2p:NTF with primers flanked by attB sites (Deal and Henikoff lab), BP reaction was performed between attB1-NTF-attB2 fragment and the vector pDONOR221 to generate the entry vector Entry-NTF (SHE1, Invitrogen, Gateway® Technology). The destination vector, pQAN, was from Dr. Quy A.Ngo, which was modified from pMOA36 containing attR sites (Grossniklaus lab). LR reaction was conducted between SHE1 and pQAN to generate the NTF containing expression vector pQAN-NTF (SHE2). The nucellus specific promoters including pAT5G14980, pAT3G52160, and AT2G03740, and the MMC specific promoters including pAT2G24500, pAT1G72320, pAT1G11270, were amplified from the genomic DNA extracted from Col-0, with primers flanked with XbaI sites. These fragments for promoters were then digested by XbaI and inserted into SHE2, which were then digested by AscI and PmeI to remove lexA minimal promoter in SHE2, thus generating pAT5G14980-NTF (SHE3), pAT3G52160-NTF (SHE4), and AT2G03740-NTF (SHE5), pAT2G24500-NTF (SHE6), pAT1G72320-NTF (SHE8), pAT1G11270-NTF (SHE9), respectively. Generation of p*KNUCKLES*-NTF (pAT5G14010-NTF, SHE10) was similar to that for SHE3-9, except that the promoter region of *KNUCKLES* was amplified with primers flanked by XbaI and AscI. p*KNUCKLES* fragment, digested by XbaI and AscI, was then inserted into SHE2.

The *KNUCKLES* promoter flanked by HindIII and BamH1 was amplified from Col-0 genomic DNA, which was digested by HindIII and BamH1, and then inserted into pCB72 to generate pKNU-EGFP (SHE11) that drives the expression of EGFP. pCB72 was from C. Baroux. The primers here used for all of these vectors construction were listed in Appendix (Table 1), while information for the vectors was shown in Table 2 (Appendix).

Transformation of *Arabidopsis* and selection of transformants

The expression vectors including SHE3, 4, 5, 6, 8, 9, 11 were transformed into *Agrobacterium tumefaciens* (GV3101) via the freeze-thaw method modified from *Höfgen et al. (1988)*. *Arabidopsis* plants were then transformed using the method slightly modified from *Bechtold* 1993 and *Clough* and *Bent* 1998. Grow healthy *Arabidopsis* plants at 18-20°C in a plant growth chamber until they are flowering. Clip first bolts to encourage proliferation of many secondary bolts. Plants will be ready roughly 4-6 days after clipping. Then prepare *Agrobacterium tumefaciens* strains for SHE3, 4, 5, 6, 8, 9, 10, and 11. Inoculate a 200mL liquid LB culture with 1mL of an overnight miniculture (2-3mL) for each strain, and keep them at 28°C for approximately 16hrs, with antibiotics to select for the binary plasmids (100µg/mL Rifampicin for GV3101, 60µg/mL Gentamycin for Ti helper plasmid selection and Kanamycin for T-DNA selection of SHE11, but 50µg/mL Spectinomycin for the others). Then spin down *Agrobacterium* (OD = around 0.8) and resuspend in 500mL 5% Sucrose solution. Before dipping, add Silwet L-77 to a concentration of 0.05% (500 µl /L) and mix well. Dip above-ground parts of plants in *Agrobacterium* solution for 2 to 3 seconds, with gentle agitation. Then place dipped plants under a plastic cover for 16 to 24 hours to maintain high humidity. Water and grow plants normally, tying up loose bolts with stakes. Stop watering as seeds become mature. Then harvest dry seeds.

Seeds were sterilized and directly sprayed to soil and grown under long-day condition (16 hours light/8 hours dark) at 18-20°C in a plant growth chamber. Spray with 20-30 mg/L Basta to the seedlings at 2-4 true leave stage for 3-4 times. The survived and healthy plants were considered as successful transformants and used for further analysis.

Screening for transformants

Young flower buds were collected from the Basta resistant transformants, and then dissected on the microscopic slide under the dissecting scope (For screening of SHE11 T1

transformants, cell membranes of the carpels were stained by FM®4-64). Add around 30ul 0.5x MS with 1M glycine, and cover with the coverslip. The dissected ovules on the slide were then checked for GFP expression in MMC by Fluorescence Microscope (DM6000) or Confocal Laser Scanning Microscope (CLSM).

MMC nuclei isolation

Collect 1g young flower buds from pKNU-nlsYFP line into 2 ml Eppendorf tube, add 1 ml Fixative Buffer (4% formaldehyde prepared freshly in Tris buffer). Rinse 2× with Tris buffer (10mM Tris, 10mM Na_2EDTA, 100mM NaCl, 0.1% Triton X-100, pH7.5). Then, transfer the flower buds to a small petri-dish, and add 500ul Nuclei Isolation Buffer (15mM Tris, 2mM Na_2EDTA, 0.5mM Spermin, 80mM KCl, 20mM NaCl, 15mM β-mercaptoethanol, 0.1% Triton X-100). Chop the flower buds into small pieces with fresh razor blade. Then pass the supernatant into 35 μm mesh filter (BD) and collect the isolated nuclei by 1.5 ml Eppendorf tube. Stain the nuclei with 4 μg/ml DAPI. Drop 20 μl "Sucrose Pillow" on the slide, and then add 20-30 μl nuclei solution onto the "Sucrose Pillow". The sucrose pillow is the solution with 100 mM Tris, 50 mM KCl, 2 mM $MgCl_2$, 0.05% Tween, 5% Sucrose. At this stage, the slide with nuclei can be checked by DM6000, or dry the slide at 4°C overnight (or store at -20°C later for further microscopy).

Results

Generation of INTACT lines for chromatin profiling

The INTACT system (Deal and Henikoff 2010) relies on two components: (i) a nuclear tag (NTF), a GFP-fusion protein targeted to the nuclear envelope containing a biotin ligase recognition peptide (BLRP), (ii) a biotin ligase (BirA). Both components have to be expressed in the target cells to tag the nuclei with biotin for further purification (Figure 1A).

- BirA line: the candidate line is expressing a hBirA::HA tagged protein under the *RPS5A* promoter (pRPS5A::hBirA-HA, Dr. Alex Boyko, Grossniklaus lab).
- pMMC:NTF and pNucellus:NTF lines: 3 nucellus specific promoters (AT5G14980, AT3G52160, AT2G03740) and 3 MMCs specific promoters (At2G24500, At1G72320, At1G11270) were used according to a published transcriptome data (Schmidt et al., 2011).

The MMC specific promoter p*KNUCKLES* (pAT5G14010) (Payne et al., 2004; Tucker et al., 2012) was also used to drive the expression of NTF (Figure 1A). The pMMC:NTF and pNucellus:NTF constructs were transformed into Col-0.

No NTF expression in the MMCs of transformants

The pMMC:NTF (SHE6, SHE8, SHE9, SHE10) and pNucellus:NTF (SHE3, SHE4, SHE5) transformants selected by Basta, were check for GFP fluorescence under epifluorescent and Confocal microscopy, however, none of these lines show GFP expression on the nuclear envelope of MMCs or nucellular cells. The lines screened were summarized as follows (Table 1):

Table 1. Lines screened for MMC or Nucellar specific NTF expression.

pMMC:NTF	Number of lines screened	pNucellus:NTF	Number of lines screened
SHE6	24	SHE3	No lines selected with Basta
SHE8	59	SHE4	29
SHE9	2	SHE5	9
SHE10	200		

Verify the activity of *KNUCKLES* promoter

The *KNUCKLES* promoter was specific and active in MMC, which can be evidenced by MMC specific conspicuous YFP fluorescence in pKNU-nlsYFP line (Figure 1B, Tucker et al., 2012). However, no GFP signals were detected in MMCs of pKNU-NTF transformants (SHE10), as shown in Table 1. For this, we tested again the activity of *KNUCKLES* promoter in driving the expression of EGFP (pKNU-EGFP, SHE11). It is of note that EGFP signals were only detected in MMCs of 5 lines in 35 Basta selected T1 pKNU-EGFP lines (SHE11), with much weaker signals in MMCs compared to that of pKNU:nlsYFP (Figure 1B, Figure 2), suggesting that *KNUCKLES* promoter is still active and specific in MMC, but with unstable and compromised activity in promoting EGFP expression. Thus, possibly failure in NTF expression on the nuclear membrane of MMCs in pKNU-NTF line is either due to the attenuated activity of *KNUCKLES* promoter in driving EGFP expression or insertion into loci surrounded by repressors that cause positional effect as no EGFP signals were detected in 30 lines of 35 Basta selected T1 lines.

Fluorescence Assisted Cell Sorting (FACS, Figure 1B)

As the INTACT lines did not work, thus FACS on the pKNU-nlsYFP line was also tested (Tucker et al., 2012). An additional line expressing YFP in nucellus nuclei using one nucellular specific promoter, selected from transcriptome date, is essential for cell sorting of nucellus nuclei (Schmidt et al., 2011). Before FACS, I tried to isolate MMC from pKNU-nlsYFP line, the nuclei were with good quality, however, rare nuclei were detected with YFP signal, as shown in figure 3, suggesting that MMC nuclei were either still embedded in surrounding tissue, and filtered out with 35 µm mesh filter, or quenched with YFP signal due to the reagents with reducing activity.

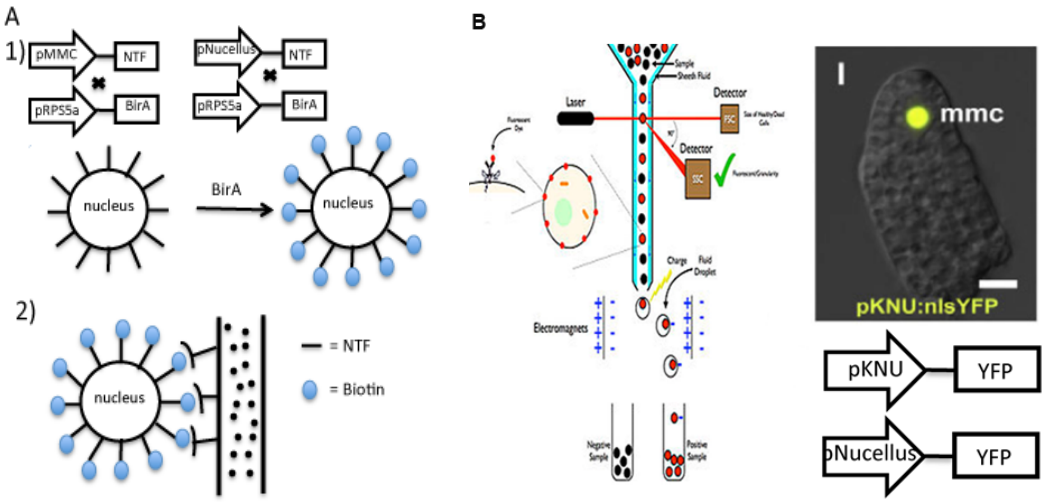


Figure 1. Two systems for isolating MMC nuclei: A) INTACT system, B) Fluorescence assisted cell sorting (FACS). This was modified from *Tucker* et al., 2012, and http://en.wikipedia.org/wiki/Flow_cytometry.

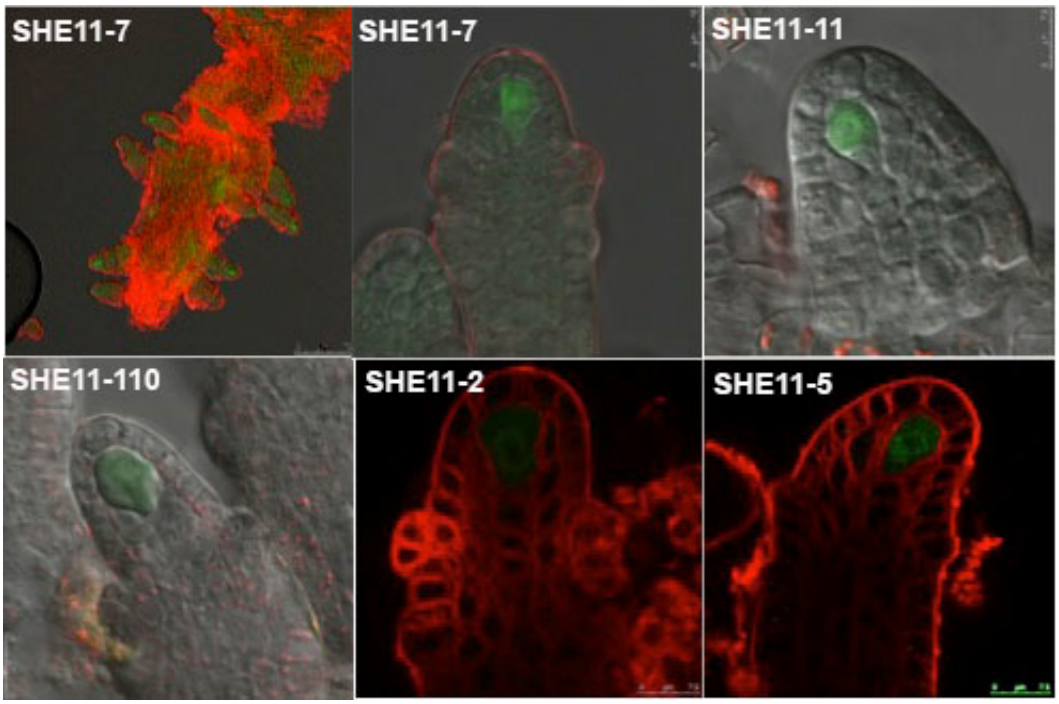


Figure 2. Weak expression of EGFP driven by *KNUCKLES* promoter in MMCs of 5 pKNU-EGFP lines (SHE11). Representative images for SHE11-7, 11, 110 are shown as the overlay of GFP and transmitted light (DIC), while SHE11-2, 5 were the overlay of GFP and FM®4-64. T1 lines were generated by me and screened by C.Baroux. Images were from C.Baroux.

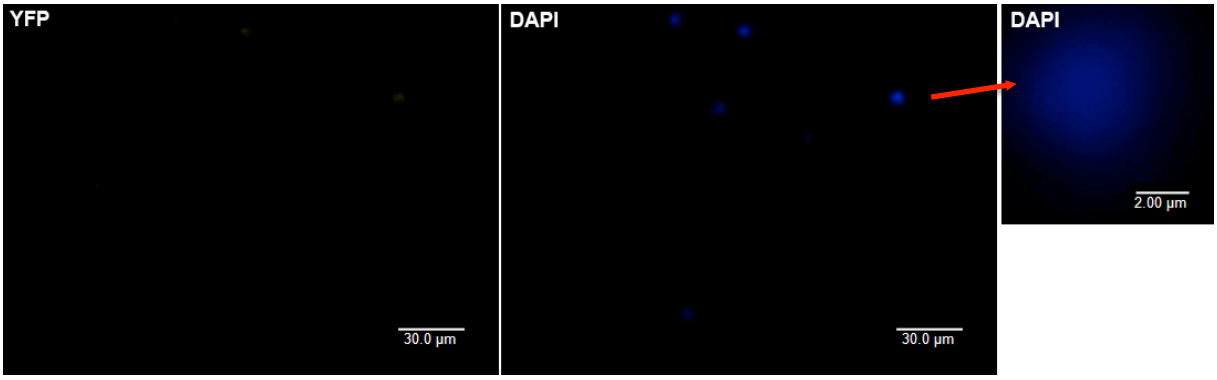


Figure 3. Isolated nuclei from carpels of pKNU-nlsYFP. The nuclei were in good quality, as marked by DAPI, but no YFP fluorescence detected in these nuclei, suggesting that the isolated nuclei were either not from MMCs, or some from MMCs with quenched fluorescence.

Discussion

By contrast to animals that derive their germline early during embryogenesis, flowering plants produce their reproductive structures and germline cells during adult development, in the flower. We recently showed that ovary cells at the somatic-to-reproductive fate transition undergo a dramatic, global chromatin reprogramming. These events are central for establishing a post-meiotic competence in forming a viable germline (She et al., 2013). *Arabidopsis* plants deficient in the histone methyltransferase SDG2 undergo improper chromatin reprogramming (She et al., 2013) and fail in deriving a viable germline (Berr et al., 2010). However, it remains unknown how the dramatic epigenetic reprogramming regulates MMCs differentiation. While genome-wide profiling maps of chromatin from MMCs that will be immunoprecipitated with antibodies against histone modifications, followed by next generation sequencing (ChIP-seq), will be essential to uncover this puzzle. However, the purification of nuclei from MMCs was hindered by technical limitations to isolate highly diluted MMCs from nucellar tissue.

The INTACT system is an elegant method for the rarest cell types profiling without isolating whole cells, which relies on the affinity purification of nuclei with the biotinylated fusion protein on the nuclear envelopes (Deal and Henikoff, 2010; 2011; Wuest et al., 2013). It has been successfully employed in the model organisms such as *Arabidopsis*, C.elegans and Drosophila, with high efficiency (100-10,000× enrichment scores) (Deal and Henikoff, 2010; 2011; Henry et al., 2012; Steiner et al., 2012; Wuest et al., 2013). Thus, we tried to test this system, in which the target nuclei express a tag on their nuclear envelope (NTF). NTF is a fusion between the GFP fluorescent reporter, a nuclear envelope protein and a biotin ligase recognition peptide (BLRP). Expression of biotin ligase (BirA) in the target cell will allow tagging the nuclei with biotin for further purification on streptavidin columns. However, none of the MMC or nucellus specific NTF expressing lines showed GFP signals on MMC or nucellus nuclear membrane (Table 1). It is noteworthy that the *KNUCKLES* promoter strongly drives the expression of YFP in MMC as shown in pKNU-nlsYFP (Tucker et al., 2012). However, we found that the EGFP signal in MMC nucleus of pKNU-EGFP line was not that strong (Figure 1), and only 5 in 35 Basta resistant lines exhibit fluorescence, suggesting that expression of GFP by *KNUCKLES* promoter is unstable, which may be one of the reasons for no NTF expression on the MMC nuclear membrane of pKNU-NTF lines. For this, fusion of a translation enhancer with NTF might be an alternative for improving stable expression of

NTF on the nuclear membrane. Or else, the replacement of GFP with RFP in NTF would enhance the fluorescence intensity on the nuclear membrane. The NTF region is integrated into the genomic DNA in pKNU-NTF transgenic line, which was proved by sequencing, while the transgenic expressed NTF protein may be degraded by the internal system as a defense against exotic proteins. Additionally, it could be either triggered by transcriptional silencing of NTF mRNA if the level of NTF transcript surpassed a certain threshold or insertion of transgenes into transcriptionally repressive loci that leads to positional effect.

FACS is based on sorting cells that are tagged with a fluorescent marker using a flow cytometry system, which is limited to cells at a relatively higher occurrence, and results in much lower output of cells compared to the INTACT system (Wuest et al., 2013). Although it may not be an elegant method for sorting the rarest cell types such as MMC, we still tried to use this method for isolating MMCs from the carpels by manually enriching the samples using the MMC specific line pKNU-nlsYFP (Tucker et al., 2012). The nuclei from the smashed carples were in good quality, but none of them was detected with YFP fluorescence. Thus, MMCs nuclei were not successfully isolated from the carpels. While difficulty in detaching MMC from surrounding nucellar cells may account for this.

Epigenome profiling of MMCs will be important for a better understanding of how epigenetic reprogramming regulates cell fate transition at a better resolution. Comparing the epigenome of MMCs to those published of vegetative or whole inflorescence tissue will be important to answer the question whether a tissue specific epigenome is established. Further, with this work we will be able discussing possible similarities or differences in epigenetic reprogramming between animal primordial germ cells and plant spore mother cells.

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6. Result Chapter IV

Chromatin reprogramming in *Arabidopsis* PMC

Abstract

Sexual reproduction in flowering plants is marked by several fate transitions: from the somatic-to-reproductive cell fate transition (SRT), from haploid spores to mature gametophyte during gametogenesis, and from gametes to double fertilization products. These processes are accompanied with extensive chromatin reprogramming, which orchestrates new cell fate acquisition. Our recent work also shows that large-scale chromatin reprogramming underlies SRT in differentiating female megaspore mother cell (MMC) in *Arabidopsis*. However, little is known about chromatin reprogramming during SRT in male germline establishment. Here, this work uncovers that specification of male pollen mother cells (PMCs) is also characterized with dynamic changes of chromatin organization, with loss of linker H1 and histone variant H2A.Z, and distinct histone modification patterns compared to that in surrounding somatic cells, suggesting establishment of a permissive epigenetic landscape in PMCs. Further analyses of mutants with misexpression of chromatin modifiers will be essential for elucidating the functional significance of chromatin dynamics in PMCs.

Introduction

Unlike animals, where their male germline is determined early in embryogenesis, plants set aside their male germline lineage after the completion of flower organ development, which is initiated by differentiation of pollen mother cells (PMCs or microspore mother cell) from somatic cells in the anthers. The male meiocytes give rise to four haploid microspores, each of them will form a binucleate pollen grain with one smaller generative cell and one bigger vegetative cell after mitosis, while the generative cell divides to give rise to two sperm cells (SCs) that will provide the paternal genetic contribution to the zygote and endosperm in double fertilization (Maheshwari, 1950).

Male gametophyte development is marked by extensive chromatin reprogramming, which is evidenced by the dimorphic epigenetic states established in the vegetative cell and generative/sperm cell. Both of these cell types are stripped of somatic H3 variants, instead only HTR5, HTR8, and HTR14 are present in the vegetative cell, while HTR5, the sperm-specific HTR10, and CENH3 are enriched in the sperm (Ingouff et al., 2007; Ingouff et al., 2010). Chromatin in the vegetative cell is decondensed, accompanied with eviction of linker H1 and enrichment of RNA PolII, but weakly distribution of H3K4me2 and H3K9ac, underlying a bivalent epigenetic landscape is established in the vegetative cell. Removal of H3K9me2 in the vegetative cell at bicellular/tricellular stage leads to disassembly of centromeric heterochromatin (Tanaka et al., 1998; Schoft et al., 2009; Houben et al., 2011). While chromatin in the sperm cell is condensed, with accumulating H3K9me2, H3K9ac and H3K4me2, but absence of RNA PolII and reduction of H3K27me3, suggesting a transcriptional quiescent state is established in the sperm cell, which is inherited from the generative cell (Houben et al., 2011). It is of note that chromatin dynamics during male gametophyte development is also reflected by the distinct DNA methylation patterns established between the vegetative cell and the gametes. The condensed sperm chromatin inherits DNA methylation patterns from the microspore, with CHH demethylation at TE loci, but retains CG and CHG methylation. By contrast, chromatin in the vegetative cell is less condensed, with remethylation of CHH contexts at TE loci, but loss CG methylation (Calarco et al., 2012). Demethylation in the vegetative nucleus results in reactivation of TE, which will generate siRNA that could transpose to reinforce TE silencing in the sperm (Slotkin et al., 2009; Calarco et al., 2012; Ibarra et al., 2012).

Thus, drastic chromatin reprogramming is associated with male gametogenesis, which plays fundamental roles in transcription regulation and TE silencing in the gametes and early embryo. However, whether chromatin reprogramming occurs even before meiosis, in the differentiating PMCs, remains elusive. The ARGONAUTE proteins are important players in epigenetic regulations involving microRNAs (miRNAs)- and small-interfering RNAs (siRNAs)-directed post-transcriptional gene silencing (PTGS) and RNA directed DNA methylation (Vaucheret, 2008). The rice AGO gene *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*) is essential for microsporogenesis, with an arrest at early Prophase 1 in the mutant lacking *MEL1* activity. Possibly, *MEL1* is important to repress somatic gene expression via small RNA directed gene silencing to promote germ cell fate acquisition as *mel1* mutant male SMCs (pollen mother cells, PMCs) carry somatic cell-type mitochondria. It is noteworthy that

some of the *mel1* male spore mother cell that arrested at leptotene or zygotene are characterized by reduced H3K9me2 intensity and altered nucleolar organizing region (NOR), suggesting chromatin reprogramming may play important roles during PMCs differentiation (Nonomura et al., 2007). Furthermore, recently we have shown that, likewise animal PGCs, chromatin reprogramming in female SMCs of flowering plants, at least in *Arabidopsis thaliana* (including drastic chromatin decondensation, changes in nucleosomal composition, quantitative alteration of the histone modification landscape) contributes to establishing competence for the post-meiotic, gametophytic fate (She et al., 2013).

Thus, the question is whether large-scale chromatin reprogramming underlies the specification of PMCs. To address this, we analyzed the chromatin modifications in differentiating PMCs, and found a similar dynamic chromatin changes to that in female MMCs, suggesting chromatin reprogramming is commonly employed during the somatic-to-reproductive cell fate transition.

Materials and Methods

Arabidopsis plants were grown under long-day condition (16 hours light/8 hours dark) at 18-20°C in a plant growth chamber. Here *Arabidopsis thaliana* accession Col-0 was used for immunostaining. H2A.Z-GFP is pHTA11:HTA11-GFP (Kumar and Wigge, 2010). Generation of H1.1-GFP and H1.2-GFP lines were described as that in *she et al., 2013*.

Immunostaining of linker H1, H3K27me1, and H3K27me3 were performed as that described for whole-mount ovule primordium immunodetection, with minor modifications (She et al., 2013; She et al., 2014). Young anthers were fixed with 1% formaldehyde and 10% DMSO in PBS-Tween (0.1%) before dissection and embedding of the anthers in 5% acrylamide pads on microscope slides. The samples were then processed by clarification (methanol/xylene), cell wall digestion, permeabilization, and 5% BSA blocking (40min to 1hr) before application of the primary for 12-14 hours, then secondary antibody for 12-24 hours at 4°C. The samples were counterstained with propidium iodide and mounted in Prolong Gold (Invitrogen). Here the primary antibodies against H1, H3K27me1, H3K27me3, as well as the secondary antibodies are all diluted by 1:200. The primary antibody against H1 was from Jerzmanowski's group, University of Warsaw, Poland (Agrisera, AS11 1801), while the other primary antibodies are from upstate.

Images of fluorescent signals in whole-mount anthers were recorded by confocal laser-scanning microscopy with a SP5-R (Leica Microsystems) using a 63× GLY lens (glycerol immersion, NA 1.4).

Results

Differentiation of *Arabidopsis* PMC in the anthers

Male reproductive lineage development begins with the differentiation of PMCs in the anther locule. In *Arabidopsis*, the archesporial cell derives from a somatic cell at sub-epidermal position in the sporangium, which then divides to form the primary parietal cell toward the exterior and the primary sporogenous cell toward the interior. The primary parietal cell further divides to give rise to four layers of anther walls, while the primary sporogenous cell produces two layers of PMCs after mitosis (Maheshwari, 1950). The differentiation of PMCs is accompanied with changes of cellular and nuclear morphology, marked by cellular enlargement and increase in nuclear and nucleolar size (Figure 1). The distinct morphology of PMCs from those of somatic cells in the anther walls suggests large-scale chromatin reorganization occurs during somatic-to-reproductive cell fate transition.

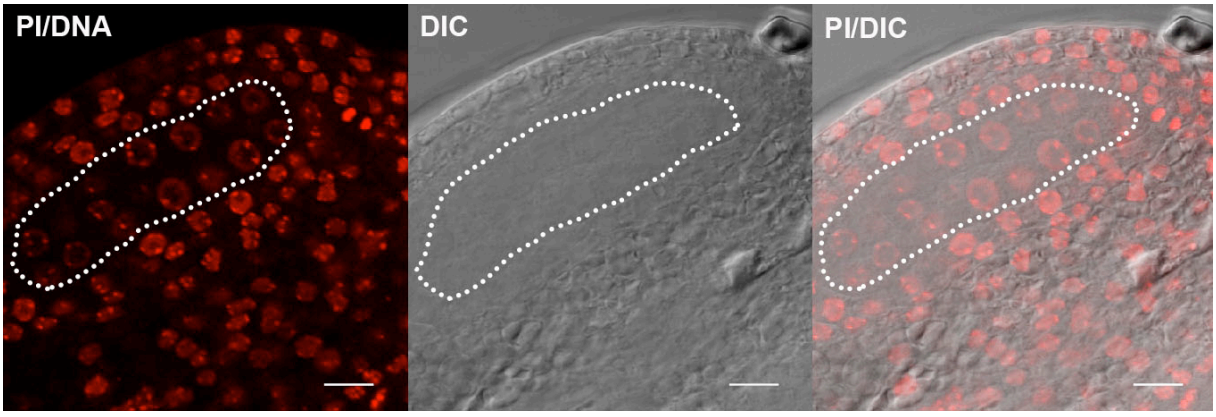

Figure 1. Specification of PMCs in the anther. Compared to surrounding somatic cells that comprising the anther walls, PMCs are marked by the enlarged cellular and nuclear size. Representative images are shown for DNA counterstaining (Propidium iodide, PI), differential interference contrast (DIC), and the DIC image overlaid with PI counterstaining. PMCs are indicated by dotted lines in white. Scale bar: 10μm.

Eviction of histone variants in PMCs

The enlarged nucleus and nucleolus indicate an open and decondensed chromatin state is established in PMCs. While linker histone H1 is the fundamental regulator of chromatin compaction (Hood and Galas, 2003). There are three canonical H1 variants encoded by *Arabidopsis* genome, including H1.1, H1.2, H1.3, with H1.1 and H1.2 widespreadly expressed during plant development (Wierzbicki and Jerzmanowski, 2005). Thus, we analyzed dynamic nuclear distribution of GFP-tagged variants in the developing anther, and found no fluorescence in PMCs of H1.1-GFP and H1.2-GFP lines, suggesting eviction of H1.1 and H1.2 in PMCs (Figure 2A, analysis by C. Baroux). This was confirmed by immunostaining on whole-mount anthers using antibody against H1, which shows eviction of H1 in PMCs, indicating that PMCs chromatin is loosen and open for further chromatin dynamics (Figure 2B). Depletion of H2A.Z in PMCs, a histone variant that leads to both activation and repression of target loci with its depletion due to increased temperature (Kumar and Wigge, 2010), was also observed, suggesting dynamic changes in histone variants (Figure 2A, analyzed by C. Baroux).

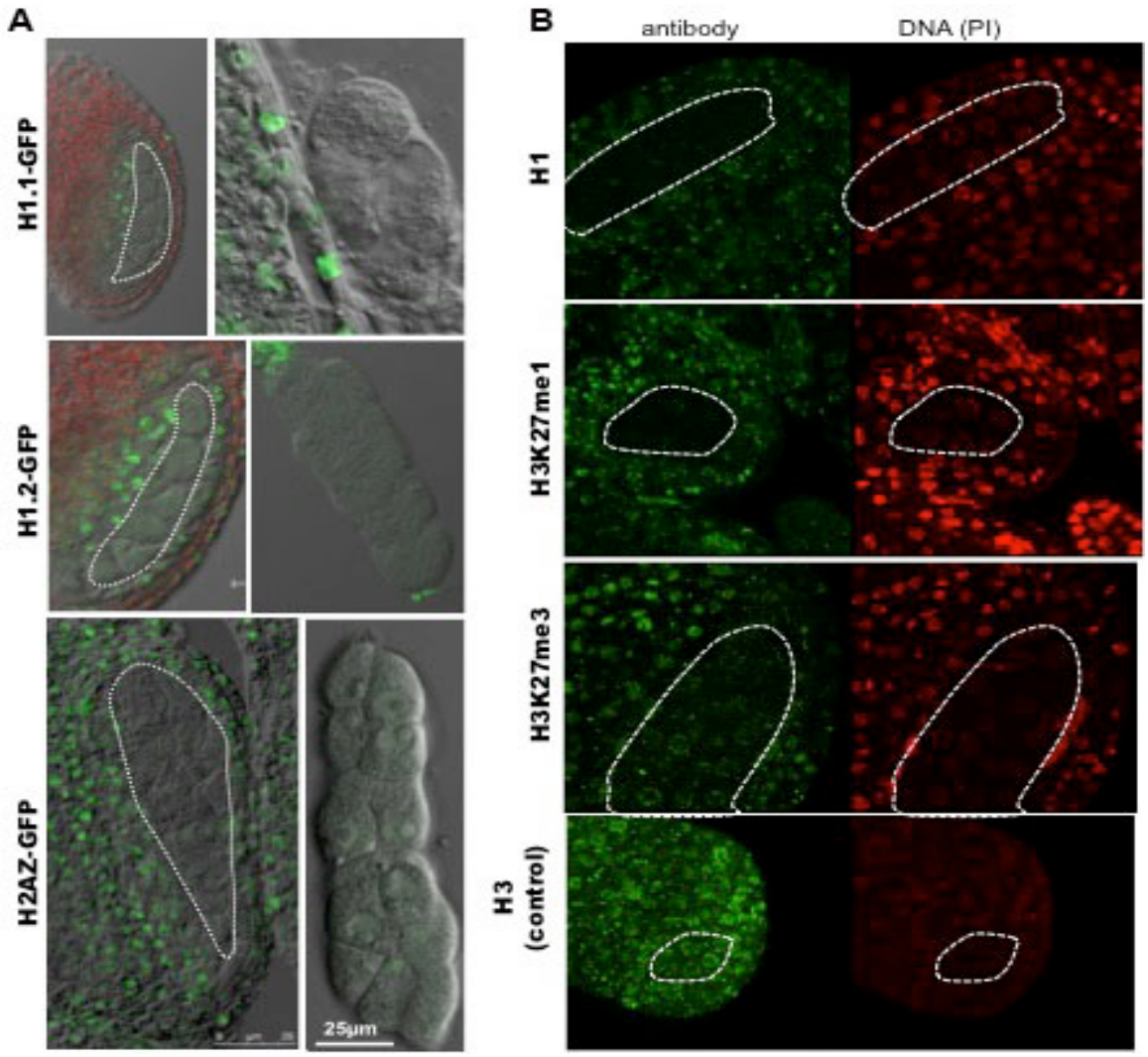

Figure 2. PMCs chromatin differs from that of surrounding somatic cells by distinct patterns of histone variants and histone modifications. (A) Eviction of H1.1, H1.2 and H2A.Z in PMCs, as illustrated by dynamic localization of GFP-tagged histone variants in differentiating PMCs. Data analyzed by C. Baroux. (B) Depletion of linker H1 and H3K27me1, as well as reduction of H3K27me3 in PMCs, as shown by immunostaining of whole-mount young anthers. Representative images are shown for the antibody, and DNA (Propidium iodide, PI), with PMCs marked by white contours.

PMCs entail distinct histone modifications

Eviction of histone variants in PMCs suggests further dynamic changes of PMCs chromatin structure, which is usually conferred by post-translational modifications of histones, as described in the general introduction part. Thus, we then analyzed histone modifications

associated with either repressive euchromatin regions (H3K27me3), or repressive heterochromatin regions (H3K27me1), whereas H3 as a control, via immunostaining with antibodies against these histone marks on young anthers. Notably, similarly to that in MMC, H3K27me1 was evicted in PMCs where H3 was constant, compared to that in surrounding somatic cells. A decrease of H3K27me3 was also detected in PMCs (Figure 2B).

Chromatin reorganization is often conferred by dynamics changes of histone modification, which in turn influence the accessibility of molecular factors to DNA, thereby regulate transcription. The distinct chromatin modifications in PMCs, which is marked by loss of the repressive marks including H3K27me1 and H3K27me3, suggest a permissive epigenetic landscape and transcriptional permissive state is established in PMCs. However, this awaits confirmation as other histone marks also influence transcription by regulating chromatin structure.

Chromatin dynamics in PMCs at the onset of meiotic Prophase I

PMCs differentiation is followed by the onset of meiotic Prophase I, which is marked by condensed chromatin. PMCs restore linker H1 at the onset of meiotic Prophase I, which may function in chromatin condensation for meiosis (Figure 3). By contrast, H3K27me3 is further reduced to the signals below detection (Figure 3). Thus compared to that in differentiating PMCs, PMCs chromatin at the onset of meiotic Prophase I entails both similar and distinct chromatin changes, which is possibly required for meiotic execution.

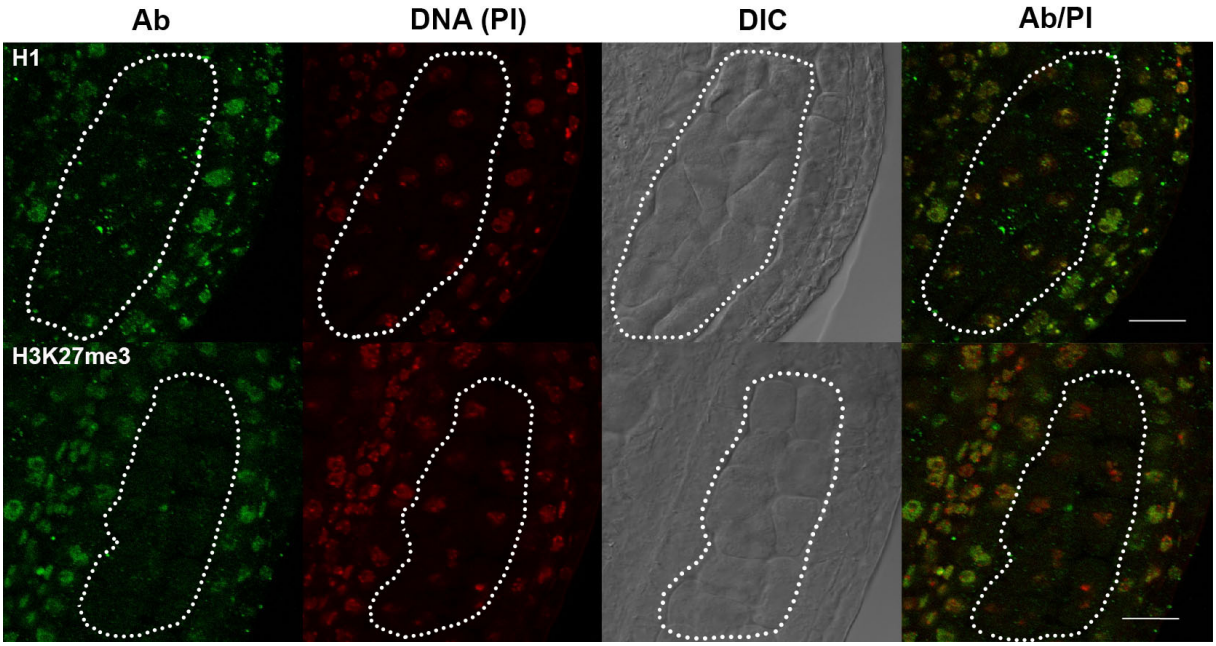

Figure 3. Reloading of linker H1 and dramatic reduction of H3K27me3 in PMCs at the onset of meiotic Prophase I. Representative images respectively for antibody (Ab), DNA (PI counterstaining), DIC, overlay of Ab with PI are shown here. PMCs, with condensed chromatin, are marked with dotted lines in white. Scale bar: 15μm.

Discussion

The work described here shows that the somatic-to-reproductive cell fate transition during male sporogensis is marked with chromatin dynamics, which is similar to that during female sporogenesis (She et al., 2013). The transient loss of linker H1 implies an open chromatin structure is established in PMCs, which is consistent with the increase of nucleus and nucleolus size. The open chromatin structure is often associated with chromatin decondensation in PMCs, which is evidenced by the erasure of the repressive mark H3K27me1 and reduction of H3K27me3. Loss of these repressive marks may be critical to release suppressed chromatin to become transcriptionally active, which possibly contributes to germline program initiation.

Differentiation of PMCs is followed by meiotic Prophase I execution that involves homologous pairing, synapsis, and recombination in most organisms. The dynamic changes of chromatin organization may serve as a regulator for meiotic prophase initiation and execution. It is of note that linker histone H1 is restored into PMCs before the onset of meiotic Prophase

I, suggesting that dynamics of linker H1 underlies meiotic Prophase I initiation in PMCs. At the onset of meiotic Prophase I, chromatin becomes condensed in PMCs, which is at least contributed by recruitment of linker histone H1. Linker histone variants deficiency results in dramatic chromosomal aberrations during male meiosis in tobacco, indicating that linker histone is essential for meiotic progression (Prymakowska-Bosak et al., 1999). Reloading of H1 in *Arabidopsis* PMCs may also function in meiotic progression. While in mice mutant with defected H3K9me2 levels, the synapsis was disrupted in meiotic prophase, thus H3K9me2 is critical for meiotic prophase progression in mice, possibly also in *Arabidopsis* meiosis as H3K9me2 seems to be accumulated in differentiating PMCs (Data not shown) (Tachibana et al., 2007).

Regarding the topic of chromatin dynamics in PMCs, intriguing questions await further investigation. One of the questions now is whether chromatin dynamics in PMCs entails further chromatin changes, analyses of other chromatin marks including H3K4me2/3, H3K9me1/2, and the histone variant like CENH3, as well as the pattern of DNA methylation, in differentiating PMCs will help to resolve this. While the information related to RNA PolII level in PMCs is important to verify the transcriptional competence that is consistent with the permissive chromatin landscape established in PMCs.

To elucidate the possible roles of chromatin dynamics in PMCs, functional analyses of mutants where chromatin modifications are affected should be involved. Here, importance of H3K27me1 eviction in PMCs will be elucidated by analyzing potential PMCs specification defects in ATXR5 or ATXR6 overexpression line, here ATXR5/6 mediate H3K27me1 deposition (Jacob et al., 2009). While by analyzing PMCs differentiation in mutants lacking H3K27 demethylase REF6 activity will be helpful to characterize the functional significance of H3K27me3 reduction in PMCs (Lu et al., 2011). Further, how changes of chromatin modifications operate the somatic-to-reproductive cell fate transition? Epigenome profiling of PMCs using antibodies against histone marks, followed by next generation sequencing will be important to identify the target loci regulated by changes of histone modifications in PMCs, which would provide a clue for uncovering the mechanisms involved in this process.

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7. Result Chapter V

Chromatin reprogramming in Rice MMC

State of the art and Aims

In flowering plants, sexual reproduction -leading to seed formation- is initiated by the development of the germinal lineage, which is established late in contrast to most animals. It is initiated by the differentiation of spore mother cells (SMCs) in male and female flower tissues, an event which marks the transition from a somatic to reproductive cell fate. Plant SMCs can be viewed as the functional equivalent of animal Primordial Germ Cells (PGCs) that will form the gametes. As plant and animal reproduction have a major impact in our society, there are tremendous research efforts in elucidating the physiological, cellular, genetic and molecular processes involved, which in turn influence reproductive success.

Epigenetic regulations are processes involving instructive biochemical modifications of the chromatin, which is constituted by the DNA and histones, the proteins around which the DNA is wrapped. Epigenetic regulations play fundamental role in diverse biological processes ranging from local transcriptional regulation in the genome to global genome regulations shaping novel expression landscapes during cellular differentiation (He et al., 2011). Differentiation of the plant germline lineage is largely under epigenetic control (Baroux et al., 2011), underlying the importance of cellular reprogramming at that developmental stage. Furthermore, we have shown that, likewise animal PGCs, plant SMCs themselves undergo large-scale chromatin reprogramming (Chapter II, She et al., 2013). In animals, such chromatin reprogramming is required for meiotic execution and for establishing pluripotency in the germline (Hajkova et al., 2008; Hackett et al., 2012). In flowering plants, at least in *Arabidopsis thaliana*, our previous work has shown that chromatin reprogramming (including drastic chromatin decondensation, changes in nucleosomal composition, quantitative alteration of the histone modification landscape) contributes to establishing competence for the post-meiotic, gametophytic fate (She et al., 2013). This suggests a broad evolutionary conservation of epigenetic modalities during plant and animal germline initiation. Yet, our work was focused on *Arabidopsis* and the question now is whether our observations are also functionally relevant in crop plants, using rice (*Oryza sativa*) as a model system.

Rice is a major crop, which feeds nearly 50% population of the world, making research on

rice sexual reproduction highly relevant at the economical and societal level. Genome wide profiling of gene expression in rice using laser microdissection of specific cell-types followed by gene microarray hybridization revealed specific dynamic gene expression, from SMC formation to the mature gametes, particularly that of genes potentially involved in epigenetic regulations (Russell et al., 2012; Kubo et al., 2013). Yet, evidence for functional significance of epigenetic processes in rice sexual reproduction remains scarce. One example is the rice mutant *mel1* lacking the function of a specific ARGONAUTE class-of-protein. The ARGONAUTE proteins are important players in epigenetic regulations involving microRNAs (miRNAs)- and small-interfering RNAs (siRNAs)-directed post-transcriptional gene silencing (PTGS) and RNA directed DNA methylation (Vaucheret, 2008). The rice AGO gene *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*), is essential for both male and female meiosis, with an arrest at early Prophase 1 in the mutant lacking *MEL1* activity. Possibly, *MEL1* is important to repress somatic gene expression via small RNA directed gene silencing to promote germ cell fate acquisition as *mel1* mutant male SMCs (pollen mother cells, PMCs) carry somatic cell-type mitochondria. It is noteworthy that some of the *mel1* male spore mother cell that arrested at leptotene or zygotene are characterized by reduced H3K9me2 intensity and altered nucleolar organizing region (NOR), suggesting chromatin reprogramming may play important roles during rice sporogenesis or gametogenesis (Nonomura et al., 2007).

However, unlike that in *Arabidopsis*, our knowledge about chromatin reprogramming during rice SMC formation is limited. Whether the chromatin dynamics characterized in *Arabidopsis* is conserved in rice remains unknown, and our goal is to elucidate this question. It will be of interest in comparing the chromatin dynamics between these two species to uncover the evolutionary divergence or conservation of epigenetic reprogramming governing plant reproduction. We initiated investigations in Rice (*Oryza sativa*) and preliminary results are introduced here.

Materials and Methods

Plant material and growth conditions

Pistills were collected from Nipponbare (*Oryza sativa* L.ssp. *Japonica*) grown under long-day condition (16 hours light, 20°C /8 hours dark, 16°C), with 70% humidity, in a rice growth

chamber.

Immunostaining in whole-mount ovule primordia

Immunostaining of H3K27me1 and H3K27me3 were performed as that described for whole-mount ovule primordium immunodetection, with minor modifications (She et al., 2014). Young carpels from rice were fixed with 1% formaldehyde and 10% DMSO in PBS-Tween (0.1%) before dissection and embedding of the ovule primordia in 5% acrylamide pads on microscope slides. Tissue processing included clarification (methanol/xylene), cell wall digestion, permeabilization, and 5% BSA blocking (40min to 1hr) before application of the primary (12-14 hours), then secondary antibody for (24-48) hours at 4°C. The samples were counterstained with propidium iodide and mounted in Prolong Gold (Invitrogen). Here the primary antibodies for H3K27me1 and H3K27me3, as well as the secondary antibodies are all diluted by 1:200. The primary antibody against H3K27me1 and H3K27me3 are from Upstate, while the secondary antibody is from Molecular Probe.

Images of fluorescent signals in whole-mount ovules were recorded by confocal laser-scanning microscopy with a SP5-R (Leica Microsystems) using a 63× GLY lens (glycerol immersion, NA 1.4).

Phenotypic analysis of rice ovule primordia

Spikelets from wild-type rice Nipponbare were dissected with hypodermic needles (1 ml insulin syringes) on the slide under dissection scope, and cleared in a solution of chloral hydrate/glycerol/ddH_2O (8:1:2), then observed under a Leica DMR microscope with a digital camera.

Results

Differentiation of rice MMC in the ovule primordium

In rice, megaspore mother cell (MMC) derives from a somatic cell at a sub-epidermal position in the ovule primordium, which is similar to that in *Arabidopsis* (Figure 1A, B). It undergoes

progressive cellular differentiation during primordium growth, with cellular and nuclear enlargment, which was observed by clearing and DNA staining (Figure 1A, B). The similar morphology of rice MMC as that of *Arabidopsis* suggests that it might be also reprogrammed, and entails specific chromatin dynamics.

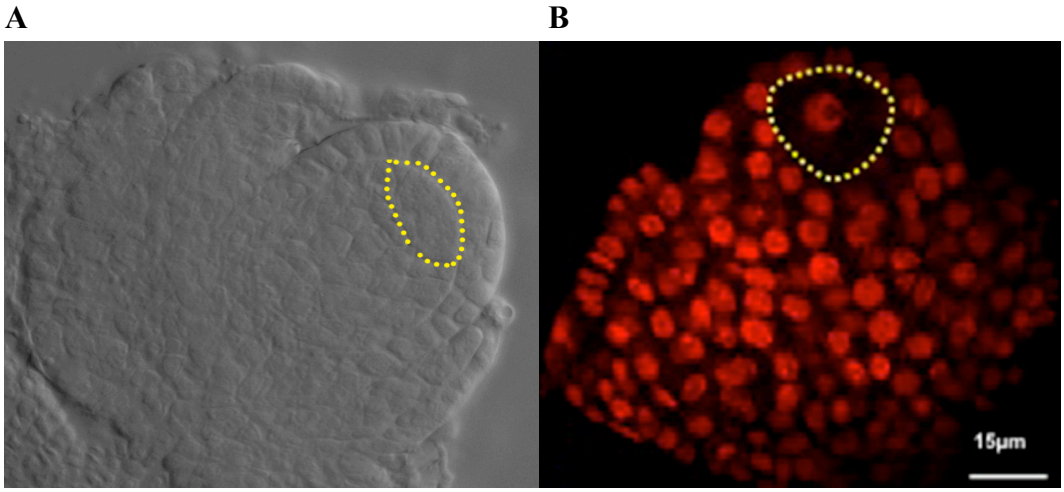

Figure 1. Differentiation of rice MMC in the ovule primordium. (A) Rice MMC originates from an enlarged subepidermal somatic cell in the nucellus, this image was acquired at differential interference contrast (DIC) phase. (B) Rice MMC is distinct from surrounding nucellar cells by its enlarged cellular and nuclear size, as shown by the 3D reconstruction of whole-mount DNA staining (propidium iodide) of the ovule. MMC is marked with dotted line in yellow.

Loss of histone modifications in rice MMC

To check whether chromatin dynamics occurs during rice MMC differentiation, we analyzed the key histone modifications including the repressive marks H3K27me1 and H3K27me3 by immunostaining on whole-mount embedded ovule primordia using antibodies against these histone marks. Interestingly, the similar trend as that in *Arabidopsis*, with loss of H3K27me3 (Figure 2A) and H3K27me1 (Figure 2B), was observed in rice MMC, indicating further chromatin reprogramming is associated with the somatic-to-reproductive cell fate transition in rice. However, this awaits confirmation by quantitative analysis of H3K27me1 and H3K27me3, as well as other histone modifications in rice.

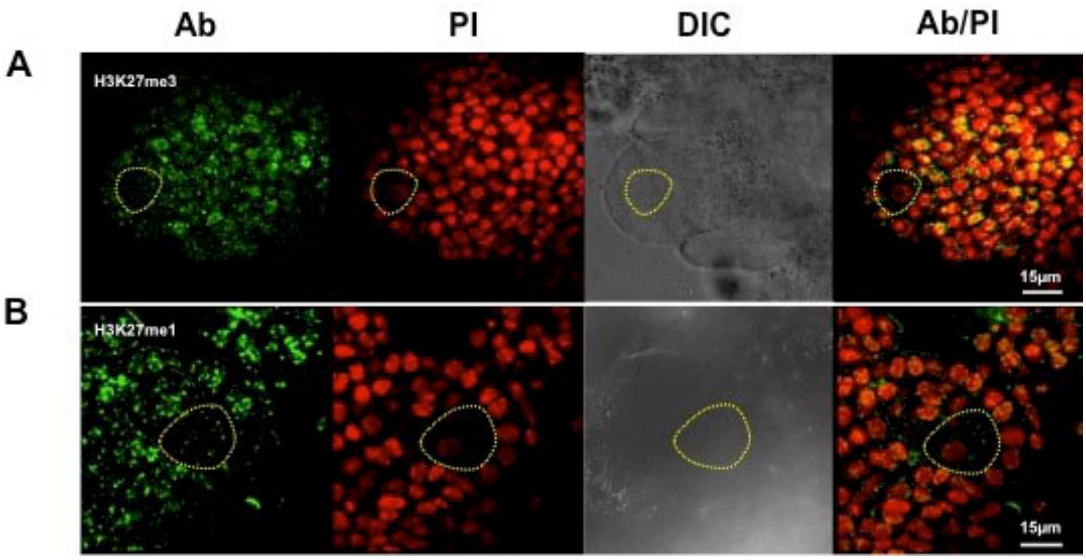

Figure 2. Loss of H3K27me3 (A) and H3K27me1 (B) in rice MMC, detected by whole-mount immunostaining in rice ovule primordia. Representative images are shown for the antibody (Ab), DNA (Propidium iodide, PI), transmitted light (DIC), and the overlay of antibody with DNA signals (Ab/PI). MMCs are marked by contours.

Discussion

Epigenetic regulations are emerging as a key processes controlling germline development in the dicot *Arabidopsis* and in maize, particularly with extensive epigenetic reprogramming taking place during the long post-meiotic gametophyte development and early embryogenesis, characterized by genome-wide dynamics of DNA methylation, histone modifications and variants. Recently, we also found out that reprogramming begins early even before meiotic prophase, which is characterized by chromatin decondensation, loss of linker histones, and changes of histone core constituents, and modifications, in differentiating female megaspore mother cells (MMCs) in the dicot *Arabidopsis*, we analyzed the nuclear organization and chromatin composition in MMCs and found large-scale chromatin dynamics in MMCs (She et al., 2013). However, little is known about that other crop plants like rice. The very preliminary results here indicate that large-scale chromatin reprogramming is also engaged in rice MMCs differentiation, which underlies a universal mechanism for somatic-to-reproductive cell fate transition.

However, to confirm this hypothesis, further investigation needs to be involved, which will pave the way for understanding the nature and function of epigenetic controls in germline

development in rice. Furthermore, using previous work from our lab and others (Grimanelli and Roudier, 2013; She et al., 2013), this study will enable us evolutionary comparisons, highlighting possible divergence or similarities in chromatin reprogramming in monocots and dicot species.

Outlook

To elucidate chromatin dynamics and its potential roles in rice MMCs, it will be necessary to analyze nuclear organization using whole-mount procedures developed in our lab that give cell-specific, quantitative information:

1- Analyze the chromatin condensation

In *Arabidopsis* female SMCs, chromatin undergoes a drastic decondensation measured by loss of heterochromatin and loss of the linker histone H1 responsible for chromatin condensation. To investigate whether this event is also taking place in rice we will specifically:

- Measure the heterochromatin content in rice ovule SMCs (whole-mount DNA staining and quantification).
- Measure the levels of histone H1 using a plant-specific antibody (She et al, 2013; from Jerzmanowski' group, University of Warsaw).

2- Analyze the chromatin modification landscape

The goal is to probe the epigenetic landscape at the nuclear-scale, by quantifying the distribution of key chromatin modifications. We will compare the MMCs to the surrounding somatic cells.

- The targeted modifications are: H3K4me2/3, H3K27me1/3, H3K9me1/2. These modifications will inform about the epigenetic status of the SMC as we did in *Arabidopsis*.
- We will check the transcriptional activity in the rice ovule SMC by quantifying the levels of Phospho-Ser2-RNA POlII, as we did in *Arabidopsis*.

3- Functional analysis of chromatin dynamics in rice SMC

Functional importance of chromatin modification in rice MMCs will be elucidated using mutant analysis. If we confirm an increase of on the heterochromatic H3K9me2 mark and a

decrease in the euchromatin silencing mark H3K27me3 as in *Arabidopsis*, we will focus on analyzing mutants affected in enzymes responsible for the deposition of this mark.

- The jmjC domain containing protein JMJ705 is required for demethylation of H3K27me3. A loss-of-function mutant is available from the Postech rice mutant database (line 1C-05110.L, http://www.postech.ac.kr/life/pfg/risd/) and shows increased H3K27me3 levels (Li et al., 2013). We will analyze potential sterility defects or SMC specification defects related to H3K27me3 levels in the ovule SMC.
- The jmjC domain containing protein JMJ706 is required for demethylation of H3K9me2. The overexpressing line available from Prof. Zhou Dao-Xiu lab (Université Paris-sud 11, France) shows decreased levels of H3K9me2 (Sun and Zhou, 2008). We will analyze potential sterility defects or SMC specification defects related to H3K9me2 levels in the ovule SMC.

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8. Review

Chromatin dynamics during plant sexual reproduction

This chapter is a draft for an invited review to Frontiers in Plant Science, special issue on "Plant Nuclear architecture" lead by Olga Pontes, Paul Fransz and Ingo Schubert. The final submitted version will be included in the final PhD thesis.

Chromatin dynamics during plant sexual reproduction

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Keywords: plant, chromatin dynamics, epigenetic reprogramming, histone modification, DNA methylation, nucleosome remodeling, small RNA, histone variants, sporogenesis, gametogenesis, fertilization.

Abstract

Plant cells have the remarkable ability to acquire new cell fates throughout their life cycle, by contrast to most animals, where cell lineages are established during embryogenesis. This ability is exemplified during sexual reproduction in flowering plants which is marked by several cell fate transitions: from the somatic-to-reproductive cell fate during sporogenesis, from the haploid spore to the gametes during gametogenesis, and from the gametes to the embryo and endosperm following double fertilization. How these distinct cell types are established in the adult plant remains a fascinating question for developmental biologists. While the molecular and genetic basis of cell specification during sexual reproduction has been studied for a long time, recent work disclosed that these processes are accompanied by large-scale chromatin dynamics. How chromatin dynamics contributes to cell fate transition during sexual reproduction and the potential biological significance of these processes will be discussed in the review.

1. Introduction

Flowering plants have a life cycle alternating between a dominant, diploid sporophytic phase and a short haploid gametophytic phase. Unlike animals, plants do not set aside a germline lineage during embryogenesis. Instead, the reproductive lineage is established late in development. The female and male reproductive lineages differentiate from somatic cells in dedicated floral tissues in adult plants. The differentiation of spore mother cells (SMCs)

engages somatic cells into a meiotic fate entailing the development of haploid, multicellular gametophytes. The gametophytes develop from the haploid spores following a limited number of mitosis and cellularization events that will give rise to very distinct cell types. In most flowering plants, the mature female gametophyte typically comprises two gametes, one egg cell and a central cell, and five accessory cells, two synergids and three antipodals; the mature male gametophyte contained in the pollen grain is composed of one vegetative cell and two sperm cells (McCormick, 1993; Yadegari and Drews, 2004). During double fertilization, the egg cell fuses with one sperm to give rise to the diploid zygote, while the central cell is fertilized by another sperm to produce the triploid endosperm. Strikingly, although genetically identical the two fertilization products share distinct developmental fates.

Genetic analyses uncovered several molecular factors responsible for cell fate establishment during plant sexual reproduction. Factors restricting male sporogenic fate include a small secreted peptide encoded by *mac1* in maize and membrane-localized leucine-rich-repeat receptor-like protein kinases (LRR-RLK) in Arabidopsis encoded by *EMS1/EXS* (*EXCESS MALE SPOROCYTES1/EXTRA SPOROGENOUS CELLS*) and *SERK1/2* (*SOMATIC EMBRYO RECEPTOR KINASE 1/2*) and their putative ligand *TPD1* (*TAPETUM DETERMINANT1*) (Sheridan et al., 1999; Zhao et al., 2002; Colcombet et al., 2005; Coleman-Derr and Zilberman, 2012; Wang et al., 2012); *mac1* also restricts the female sporogenic fate in ovules (Sheridan et al., 1999). Conversely, in Arabidopsis, the transcription factor SPOROCYTLESS/NOZZLE (SPL/NZZ) positively regulates MMC differentiation (Schiefthaler et al., 1999; Yang et al., 1999; Balasubramanian and Schneitz, 2000) through the indirect activation of *WINDHOSE1* (*WIH1*) and *WIH2* via the WUSCHEL (Koszegi et al.) transcription factor, whereby *WIHs* encode small uncharacterized peptides interacting with the tetraspanin-type protein TORNADO2 (Lieber et al., 2011). Another class of factors controlling the sporogenic fate belong to epigenetic regulators: on the one hand a non-autonomous RNA-mediated DNA methylation (RdDM) pathway acts to restrict the MMC fate in maize and Arabidopsis (Olmedo-Montfil et al, 2010; Singh et al, 2011), a function also shared by an MMC-specific RNA helicase, MEM, in Arabidopsis (Schmidt et al, 2011), while the ARGONAUTE-class of protein MEL1 positively regulates the meiotic fate of the MMC in rice (Nonomura et al., 2007). Factors controlling gametic fate in the female gametophyte include the MADS-box family of protein AGL80 and AGL61/DIANA, positively regulating

central cell fate in Arabidopsis, while the egg fate is restricted in the female gametophyte by a specific function of the splicesome machinery contributed by *LACHESIS* (*LIS*), *GAMETOPHYTIC FACTOR1* (*GFA1*), *CLOTHO* (Agger et al., 2007), *ATROPOS* (Latos-Bielenska and Vogel, 1992; Drews and Yadegari, 2002; Portereiko et al., 2006; Coury et al., 2007; Gross-Hardt et al., 2007; Steffen et al., 2007; Bemer et al., 2008; Moll et al., 2008; Steffen et al., 2008; Bemer et al., 2010). Furthermore, egg cell fate can be promoted ectopically by expressing the plant-specific transcription factors RKD1 and 2 (Koszegi et al., 2011). In addition to the activity of those cell specific factors, the gametic fate is controlled by non-autonomous signals, such as the secreted peptide ZmEAL1 in maize restricting boundaries between central cell fate and antipodal cell fates (Marton et al., 2005; Krohn et al., 2012).

In this review we propose to consider sexual reproduction as a succession of differentiation events whereby sporogenic, gametic and embryonic fates are established *de novo* and would like to focus particularly on chromatin dynamic events underlying these cellular fate transitions.

In multicellular organisms, genome expression is modulated in part by the chromatin structure that influences the accessibility and processivity of the transcription machinery (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Butler and Dent, 2012). The basic building block of chromatin is the nucleosome, which is composed of approximately 147bp of DNA wrapped around an octamer particle (containing two molecules of each of the core histones H2A, H2B, H3 and H4). The N-terminal tail of core histones can be subject to various posttranslational modifications, including methylation, acetylation, ubiquitination, phosphorylation, glycosylation, and sumoylation, which in turn modify the chromatin structure (Strahl and Allis, 2000). Chromatin compaction is further modulated by linker histones (H1), binding the linker DNA to stabilize the “beads-on-a-string” substructures (Hood and Galas, 2003). Two manifestations of chromatin can be discerned: an open, transcriptionally permissive state, and a compact, transcriptionally repressive state. Large-scale manifestations of these two chromatin states are microscopically visible in the nucleus as euchromatin and heterochromatin, respectively. Although heterochromatin is often viewed as silent, transcriptional activity particularly at repeat regions is part of a self-reinforcing mechanism of heterochromatin formation (Grewal and Elgin, 2007). In plants, taking

Arabidopsis as a model system, both euchromatin and heterochromatin domains are enriched in distinct chromatin modifications. At the cytogenetic level, while heterochromatin is typically enriched in DNA methylation, H3K9me1/2, H3K27me1/2 and H4K20me1 (Fransz et al., 2006), euchromatin is characterized by the presence of bivalent instructions such as those associated with a transcriptionally repressive state (H3K27me3) and those associated with a transcriptionally permissive state (H3K4me2/3, H3K9me3, H3K36me3, H3K56Ac, and H2Bub) (Roudier et al., 2011). The genome-wide distribution of histone and DNA methylation marks along the genome is described by chromatin profiling methods. These approaches revealed that, in somatic tissues both repressive and permissive marks rarely co-exist on the same loci, and that their differential combination within promoter or core gene regions indexes distinct chromatin states (Roudier et al., 2009). Moreover, DNA methylation is observed in three distinct sequence contexts (CG, CHG, CHH) that are distinctively enriched with gene bodies (CG) or repeat regions (CHG, CHH) (Chan et al., 2005). Histone modifications and DNA methylation are set and maintained by a cohort of enzymes, with complex interplay between themselves and chromatin remodelers but also with small RNAs acting as *trans* signals that reinforce heterochromatic states (Tariq and Paszkowski, 2004).

Heterochromatin domains are cytogenetically referred as to chromocenters; they contain rDNA, transposon, centromeric and pericentromeric repeats while euchromatin domains are composed of the distal chromosome arms deployed as rosette loops around chromocenters at interphase (Fransz et al., 2002). Additionally, chromosome organization at interphase is characterized by the formation of chromosome territories arranged in a random fashion in somatic Arabidopsis cells (Pecinka et al., 2004), although the regular spacing of chromocenters indicates spatial constraints among chromosomes (Andrey et al., 2010). While specific chromosomal associations could not be revealed by cytogenetic approaches (Pecinka et al., 2004), chromosome capture-based interaction mapping revealed multiple sites that may associate regions sharing similar chromatin states among distal chromosomal regions (Grob et al., 2013).

Chromatin dynamics refer to processes that modify the organization of eu- and hetero-chromatin domains, the distribution of genomic sequences within these domains, the arrangement of chromosome territories, and the distribution of the chromatin proteins and histone modifications. How chromatin dynamics underlie genome expression, or vice versa,

particularly during cellular differentiation remains largely unknown. The aim of this review is to discuss the emerging concept that chromatin dynamics contributes to the establishment of new cell fates during sexual reproduction, and probably to the resetting of the epigenome to a ground-state towards pluripotency in the gametophyte and totipotency in the zygote.

2. Large-scale epigenetic reprogramming during sporogenesis

In flowering plants, sexual reproduction begins with sporogenesis where spore mother cells (SMCs) are differentiated in dedicated floral organs. Female SMCs, also called megaspore mother cells (MMCs) differentiate within ovule primordia formed in the gynoecium. In Arabidopsis, a single sub-epidermal cell at the distal end of each ovule primordium enlarges and forms the archesporial cell which in turn directly develops into the MMC (Maheshwari, 1950). This pattern varies among species, whereby in some, the archesporial cell undergoes division to give rise to several MMCs (Maheshwari, 1950). The MMC undergoes meiosis to produce four haploid spores while only one survives to form the functional megaspore. Male SMCs, also called pollen mother cells (PMCs), or microspore mother cells, differentiate within the sporangium formed in the anther locule. In Arabidopsis, the hypodermal cell in the sporangium enlarges to form the archesporial cell which then divides to generate the primary sporogenous cell towards the inside and the primary parietal cell in the outside, the sporogenous cell undergoes mitosis to give rise to PMCs, while the primary parietal cells forms four layers of walls surrounding PMCs via periclinal and anticlinal divisions comprising endothecium, middle layers, and tapetum (Maheshwari, 1950). Male sporogenesis is completed after meiosis resulting in four viable haploid microspores.

The observation that the sporogenous tissue is of meristematic nature and competent to derive multiple SMCs in certain species or mutant backgrounds (see above) did not predispose to the thinking of an abrupt cellular identity transition but rather a cellular selection process within a competent tissue (Feng et al., 2013). Clearly, however, large-scale epigenetic mechanisms are controlling sporogenic fate restriction and involve both *trans* epigenetic signaling via a non-autonomous siRNA-silencing pathway as well as DNA and chromatin modifications (reviewed in Baroux et al., 2011, Grimanelli and Roudier, 2013, Guitterez and Dickinson, 2012). Here, we would like to review more particularly epigenetic

events occurring and contributing locally to the somatic-to-reproductive transition taking place during sporogenesis. Specific chromatin dynamics related to meiotic execution is described elsewhere in this issue (Plant Meiosis—Global approaches).

2.1 Chromatin dynamics during SMC differentiation

Visible changes in nuclear morphology during MMC differentiation were reported on early drawings or micrographs with clear increased nuclear size and nucleolar enlargement (Cooper, 1937; Schulz and Jensen, 1981; Armstrong and Jones, 2003; Sniezko, 2006) compared to the surrounding nucellar cells, which, in the light of our current understanding suggested large-scale chromatin reorganization. Nuclear swelling and chromatin decondensation in differentiating MMC was recently confirmed and quantified. Interestingly, it correlates with the depletion of canonical linker histones and the concomitant, yet progressive reduction in heterochromatin content (she et al., 2013). That H1 depletion is the earliest event of MMC differentiation at a stage where cellular differentiation is barely visible strongly suggests a causal link between chromatin dynamics and the somatic-to-reproductive fate transition in this cell. Following this event, the MMC chromatin undergoes further, biphasic changes in histone modifications, and nucleosomal remodeling. Chromatin dynamics, occurring along with a long meiotic S-phase, seems to establish a transcriptionally permissive state. This is suggested by a quantitative increase in the permissive-associated mark H3K4me3, and the reduction of repressive-related marks including H3K27me1, H3K27me3, and H3K9me1 in MMCs, compared to that in surrounding nucellar cells (she et al., 2013). However, decreasing levels of Ser2-phosphorylated RNA PolII and H4Kac16 indicated a moderate transcriptional competence. Thus, possibly, chromatin reprogramming in the MMC may establish a transcriptionally poised state.

The events described in the MMC are reminiscent of those observed in mouse primordial germ cells (PGCs) that can be seen as functional equivalent of plant SMCs: mouse PGCs undergo large-scale chromatin reprogramming characterized by chromatin decondensation, DNA demethylation, depletion of linker histone, histone replacement, and extensive erasure of the histone marks like H3K9me2, H3K9ac, H3K9me3 and H3K27me3 (Hajkovaa et al., 2002; Hajkova et al., 2008).

Whether pre-meiotic reprogramming of the DNA methylation landscape occurs, in the MMC, remains a fundamental question to address. Post-meiotic reprogramming has been suggested largely based on the expression dynamics of DNA methyltransferases in the female gametophyte (see section 3.3). Yet the specific impact on the actual gametic epigenome remains unknown. Possibly, given their mechanistic link with DNA methylation, H1 and H2A.Z depletion may enable profound remodeling of the methylome already in the MMC (Wierzbicki and Jerzmanowski, 2005; Kumar and Wigge, 2010; Zemach et al., 2013). Resolving the genomic loci targeted by those epigenetic reprogramming events, at the DNA or histone modification level, is the next challenge to address. Yet the techniques that would enable MMC-specific chromatin profiling are not yet established.

The mechanisms controlling chromatin reprogramming in the MMC are likely to be diverse, including both active and passive process. For instance, proteasome-mediated degradation controls histone variants eviction such as H1 (She et al., 2013) and possibly H2A.Z too. Yet upstream modifications such as phosphorylation, ubiquitinylation or citrullination may contribute to destabilize these variants (Contreras et al., 2003; Christophorou et al., 2014). Furthermore, some changes in histone modifications may be coupled with replication: the reduction in H3K27me3 levels (relative to the increasing DNA content) may be caused by incorporation of new, non-modified nucleosomes during DNA replication. This, however, does not hold true for marks such as H3K4me3 and H3K9me2 that do show a relative increase during MMC differentiation and are likely involving the activity of chromatin modifying enzymes. Yet the process may still be mechanistically coupled: it is noteworthy that H3K9me2 increases at chromocenters at stages where DNA replication is mostly detected in these domains while H3K4me3 increases in euchromatin at later stages where DNA replication is mostly detected in this nuclear compartment (She et al., 2013). Finally, we may speculate that part of the chromatin dynamics may be mediated *in trans* as suggested by the large representation of small-RNA silencing effectors in the MMC transcriptome (Schmidt et al., 2011).

By contrast, chromatin dynamics events underlying PMC differentiation in the anther are barely known. Yet, similar to MMCs, PMC nuclei enlarges in the male sporogenous cells compared to the surrounding tapetum in different species (Maheshwari, 1950). The finding that transposable elements become expressed in PMCs may further suggest decondensation at

heterochromatin loci (Yang et al., 2011) like in MMCs. In addition, H1 linker histones are dynamically phosphorylated –hence potentially destabilized- during the meiotic S-phase of wheat meiocytes (Greer et al., 2012). Possibly, PMC chromatin undergoes a selective replacement of histone H1 with notably the incorporation of a specific variant, resembling that of mouse testis (Sasaki et al., 1990). Collectively, these observations suggest that large-scale chromatin dynamics may operate during PMC fate establishment similar to that in MMCs, but detailed investigations remain necessary to confirm this proposal.

2.2 Functions for chromatin dynamics in the SMCs.

2.2.1 Preparation for meiosis execution.

The differentiation of SMCs is followed by meiotic execution. Events of the meiotic prophase I include homologous chromosome pairing, synapsis, and recombination. In mice, H3K9me2 deposition is critical for synapsis and in yeast, H3K4me3 marks meiotic recombination initiation sites and regulates double strand DNA breaks (Tachibana et al., 2007; Borde et al., 2009; Kniewel and Keeney, 2009). H3K9me2 and H3K4me3 enrichment in the chromatin of plant MMCs during the meiotic S-phase but also during prophase I (She et al., 2013) may suggest a similar role for these marks in synapsis and recombination initiation. Furthermore, the role of DNA methylation in determining the recombination landscape in Arabidopsis meiocytes (Melamed-Bessudo and Levy, 2012; Mirouze et al., 2012) may be contributed by H1 and H2A.Z dynamics in the MMC, two histone variants shown to influence DNA methylation patterns in Arabidopsis (Wierzbicki and Jerzmanowski, 2005; Zemach et al., 2013). But whether these epigenetic marks directly instruct the meiotic machinery is not known. Alternatively, an intuitive interpretation of chromatin dynamics in the MMC is to enable the expression of meiotic genes and the repression of the mitotic pathway. For instance, H1 depletion in yeast is a prerequisite to activate meiotic effectors, and H3K27 demethylation at key developmental genes in mouse is also essential to meiotic progression (Agger et al., 2007; Bryant et al., 2012). Although this is a plausible function for H1, its eviction in the MMC likely plays a role beyond meiosis execution since ameiotic *ago9* MMC resume similar chromatin dynamics than in meiotic MMCs (She et al., 2013).

2.2.2 Repression of the somatic program.

The SMC fate is not inherited, but is established locally within a niche of somatic cells in floral sex organs. Intuitively, SMC specification may thus require to exit the somatic program. In rice, *MEL1* encodes an AGO protein specifically expressed in SMCs before meiosis. Most SMCs could not complete sporogenesis and arrested at early meiosis in the loss-of-function mutant, suggesting that MEL1 is important for switching from a mitotic to a meiotic program, a prerequisite for the somatic-to-reproductive cell fate transition. Possibly as well, *MEL1* may contribute to repress other somatic features as *mel1* mutant PMCs harbor somatic type of mitochondria (Nonomura et al., 2007). In Arabidopsis, the AGO9 protein is expressed in the epidermal nucellar cells from the ovule primordia. Loss-of-function of AGO9 and other members acting in a small RNA-mediated gene silencing pathway (*suppressor of gene silencing 3* (*sgs3*) and *rna dependent rna polymerase 6* (*rdr6*)) leads to supernumerary MMCs, suggesting that the germline fate is restricted to one single cell via a non cell-autonomous small RNA pathway. It has been proposed that TEs derived small RNAs in epidermal cells may migrate to repress TEs in germ cells, which is crucial for germline fate specification (Olmedo-Monfil et al., 2010). In maize, AGO104, which belongs to the same AGO-clade as Arabidopsis AGO9, specifically accumulates in the nucellar cells of ovule primordium during sporogenesis. MMCs lacking *ago104* activity fail to undergo meiosis, resulting in unreduced (diploid) embryo sacs. Transcriptional profiling in the *ago104* mutation suggests that it represses somatic gene expression in a non-cell autonomous way (Singh et al., 2011). Collectively, the above studies allow to speculate on a non-cell autonomous, small-RNA mediated repression of the somatic cell fate during SMC specification. Interestingly, this situation is reminiscent of the animal germline which differentiation requires the inhibition of the somatic transcriptional program, partially relying on piwiRNA-mediated silencing (Nakamura et al., 2010).

2.2.3 TE silencing during sporogenesis?

Transmitting the genetic information to the next generation without accumulated mutations is a considerable challenge for sexually reproducing organisms. Transposable elements (TE) are potentially mobile sequences within the genome that pose a threat to genome integrity.

Epigenetic reprogramming during germline formation in animals, during sporogenesis in plants, is a potential risky window for TE to escape silencing. Both plants and animals have evolved different strategies to restrict TE activity, particularly in the germline (reviewed in Bao and Yan, 2012, see [3.2.2]). Chromatin decondensation, loss of heterochromatin and genome-wide remodeling of the epigenetic landscape during MMC, and likely PMC, specification in plants create a favorable environment for TE escape, thus control mechanisms are likely in place for restricting TE activity in these cells. In somatic plant cells, TEs are kept silenced via an RNA dependent DNA Methylation (RdDM) pathway, with 24nt long siRNA targeting DNA and H3K9 methylation at TE loci (Xu et al., 2013). In the MMC, despite a very low heterochromatin content (10.51% compared to 32.3% of somatic cells), the remaining chromocenters are highly enriched in H3K9me2 (She et al., 2013), whereby the immunostaining signals largely overcome the chromocenter foci. This suggests the possibility that TE silencing is reinforced although heterochromatin domains are not maintained. Furthermore, TE silencing could be mediated *in trans* by siRNAs produced by the surrounding, somatic cells of the nucellus (Olmedo-Montfil et al., 2010). Plants deficient in RdDM-mediated silencing are unable exerting a control on TE proliferation when the parental plant was subjected to heat stress and transmit novel TE copies to their progeny. Genetic analyses suggested that this control normally takes place in the floral tissue and not during gametogenesis (Ito et al., 2011). That heat-activated TEs proliferate during chromatin reprogramming in the MMC of RdDM-deficient nucellus respectively, is the most plausible explanation. Consistent with this, the transcriptionally activated retrotransposon, *EVADE*, was shown to be actively, maternally suppressed via an siRNA –mediated heterochromatin pathway before meiosis (Reinders et al., 2013) suggesting further a siRNA-based mechanism to doom TE activity during chromatin reprogramming in the MMC.

2.2.4 Epigenetic reprogramming towards pluripotency.

Sporogenesis achieves the formation of a haploid, pluripotent spore, which will generate several, distinct cell types upon gametophyte development. It has been proposed that chromatin reprogramming in the MMC contributes to establish competence to the gametophytic, pluripotent development of the spore. This proposal is based on the analysis of

mutants forming ectopic, ameiotic gametophytes in the ovule (*ago9*, Olmedo-Monfil et al., 2010) and the *sdg2* mutant that lost female gametophytic competence (Berr et al., 2010), whereby chromatin dynamics was ectopically expressed (concerning H1 eviction, H3.3 incorporation, H3K27me1 and H3K27me3 reduction) and altered for H3K4me3 levels in the MMC, respectively. Consistent with this, gametophytically expressed genes including *NZZ/SPL* are down-regulated in ovules lacking the function of the H3K4me methyltransferase SDG2 (SET DOMAIN REARRANGED GROUP2).

Although a systematic functional dissection and a challenging, single-cell epigenome profiling remain to be done, large-scale chromatin dynamics in the MMC likely enables reprogramming the epigenetic landscape to prime a gametophytic developmental program. This situation is again highly reminiscent of that in animals whereby epigenetic reprogramming in PGCs establishes a ground-state epigenome and alleviates barriers against pluripotency in the germline (Yamaji et al., 2008; Hajkova, 2011; Hackett et al., 2012). Specifically, it would be interesting to test whether H3K27 demethylation in the MMC underlies transcriptional derepression of gametophytic genes, similar to the derepression of pluripotency genes in mice and human, mediated by the H3K27 demethylase Utx (Mansour et al., 2012). The only H3K27 demethylase characterized so far in Arabidopsis, REF6 (Lu et al., 2011) does not seem involved in this process (She et al, 2013); thus determining the possible role of H3K27me3 on gametophytic gene expression awaits the elucidation of the mechanisms by which the MMC chromatin is depleted of H3K27me3.

3. Chromatin dynamics during gametogenesis

In plants, gametogenesis is the last step of gametophyte development. The gametes are differentiated, together with accessory cells, within multicellular male and female gametophytes developing mitotically from a single spore in most flowering plant species. In both genders, the establishment of distinct cell fates from genetically identical haploid cells is underlined by distinct chromatin dynamics in gametes and accessory cells.

3.1 Chromatin dynamics during male gametogenesis

Microgametogenesis begins with an asymmetric and atypical mitosis in the microspore, resulting in the formation of a large vegetative cell engulfing a smaller generative cell. The vegetative cell arrests at G1-phase, while the generative cell undergoes another mitosis to produce two sperm cells. The vegetative cell serves the function of delivering the gametes towards the ovule during fertilization. The structurally and functionally different cell types are marked by their dimorphic chromatin states. The sperm cells harbors highly condensed chromatin, while the chromatin in the vegetative cell is decondensed, correlated with linker histone H1 depletion (McCormick, 1993; Tanaka et al., 1998).

In the vegetative cell, depletion of the repressive mark H3K9me2 at the bicellular and tricellular stages indicates a transcriptionally permissive epigenetic landscape is established, which is supported by the enrichment of Ser5-P-RNA PolII. Yet, H3K4me2 and H3K9ac, two permissive marks, are also largely depleted (Houben et al., 2011), suggesting that transcriptional competence is established independently of these usual modifications. Additionally, the vegetative nucleus also undergoes centromeric heterochromatin decondensation, with dispersed 180-bp centromeric repeats (180CEN) possibly caused by the absence of the SWI/SNF-family of chromatin remodeler DDM1 (DECREASE IN DNA METHYLATION 1) in this cell (Soppe et al., 2002; Probst et al., 2003; Schoft et al., 2009). A consequence from this chromatin state is the massive transcription of transposable elements (TE) generating in turn TE-specific small-RNAs (Slotkin et al., 2009).

By contrast, transcriptional activity is almost undetectable, based on immunolocalization of Ser5-P-PolII, in the generative and sperm cells (Houben et al., 2011), although a large amount of transcripts are detected in those cells (Borges et al., 2008). This repressive transcriptional landscape may be partly contributed by enhanced H3K9me2 levels, particularly at heterochromatin loci. However, and paradocially, the sperm chromatin is enriched in the transcriptionally permissive H3K4me2 and H3K9ac, while globally depleted in the repressive mark H3K27me3 (Houben et al., 2011). Collectively, it suggests that the sperm chromatin acquires a poised state as in the animal germline.

Male gametogenesis is also accompanied by changes in the histone H3 variant repertoire, with distinct patterns established between the sperm and the vegetative cells. While both cells are devoid of the somatic H3.1 variants, they contain each a specific repertoire of H3.3

variants: the chromatin of the vegetative cell includes few canonical H3.3 variants (HTR5 and HTR8) an unusual variant HTR14, while the sperm chromatin contains HTR5 and a sperm-specific variant (HTR10) (Ingouff et al., 2010). A dynamics of core histone variants is also described in Lily pollen, with the specific incorporation in the generative cell of gH2A, gH2B, gH3 -which shares common structural properties with Arabidospsis CENH3- and the selective depletion of somatic H1 in the vegetative cells (Tanaka et al., 1998; Xu et al., 1999; Ueda et al., 2000).

Chromatin dynamics during male gametophyte development is also reflected by the distinct DNA methylation patterns established between the vegetative cell and the gametes, which can be traced back to the microspore stage before mitosis I. Comparatively to somatic cells, the microspore chromatin is devoid of CHH methylation mostly from retrotransposon loci. Gametogenesis entails antagonist changes in the sperm and vegetative cells: while the sperm cells inherit the CHH DNA methylation patterns from the microspore, with more pronounced depletion, the vegetative cells restore CHH methylation at TE loci. In contrast, CG methylation is globally retained in the sperm cells, but depleted from a subset of TE loci and intergenic regions in the vegetative cell. While CHG methylation is generally higher in the vegetative cell, albeit depleted from the same demethylated CG TE loci (Calarco et al., 2012; Ibarra et al., 2012). This profound, dimorphic remodeling of DNA methylomes during microgametogenesis is likely a consequence of differential activity of key factors in the gametes and vegetative cell: the *de novo* DNA methyltransferase DRM2 and the 24nt siRNA-based machinery, that normally act together in establishing and maintaining CHH methylation, respectively, and the DNA glycosylases DEMETER (DME) and REPRESSOR OF SILENCING 1 (ROS1) enabling CG demethylation via a base-pair excision-repair process (Morales-Ruiz et al., 2006; Law and Jacobsen, 2010; Calarco et al., 2012).

Whether DNA methylome reprogramming is a cause or consequence of large-scale chromatin dynamics is unclear. Possibly, however, depletion of H1 linker histones and of the chromatin remodeler DDM1 in the microspores (Tanaka, 1991; WS, CB, unpublished) may underscore a mechanistic link with DNA methylation reprogramming (Wierbiscki and Jerzmanowski, 2005; Zemach et al., 2013).

3.2 Functions of chromatin dynamics during male gametogenesis

3.2.1 Epigenetic reprogramming in sperm cells

The sperm cells acquire a repressive chromatin state and an apparent transcriptional quiescence. It has been proposed that this situation serves a similar function than in the animal germ cells, whereby epigenetic reprogramming reset parental genomic imprints and poise developmental gene expression as a prequisite to establish totipotency in the zygote (Seydoux and Braun, 2006). Specifically, MET1 activity in the sperm cells may act in maintaining methylation imprints for instance at the FWA and FIS2 loci that will be inherited to the fertilization products (Saze et al., 2003; Jullien et al., 2006).

3.2.2 TE silencing in sperm cells involving siRNA *trans* signaling

The problem of maintaining genome integrity in the germline has been exposed in 2.2.3. In animals, the requirement of a TE control in the germline is restricted to primordial germ cell development and meiosis (Bao and Yan, 2012), since the meiotic product directly produces the mature gamete. In plants, however, the mitotic developmental phase of the gametophyte, following meiosis, imposes the necessity to prolong a control over TE activity until the mature gametes. It has been suggested that TE activity in the vegetative cell of the pollen generates siRNA templates acting *in trans* on the sperm cells' chromatin to maintain TE silencing (Slotkin et al., 2009), although that reservation has been emitted regarding the possible transport of siRNA from the vegetative cell to the sperm cell.

Unlike sperm cells, the vegetative cell doesn't contribute to the next generation. Yet, this companion cell seems to influence the epigenetic setup of the sperm cells. The current model involves TE-derived siRNAs produced from the vegetative cell to act *in trans* in the sperm cells' chromatin to reinforce TE silencing. The companion cell would thus provide a process of genome integrity maintenance in sperm cells that are transcriptionally silent and thus unable to provide the effectors of TE silencing (Schoft et al., 2009; Slotkin et al., 2009; Calarco et al., 2012; Ibarra et al., 2012).

3.3 Chromatin dynamics during female gametogenesis

The functional megaspore undergoes three rounds of mitosis and a final cellularization process to give rise to the female gametophyte harboring two gametes, the egg cell and the central cell, as well as five accessory cells, two synergids assisting the fertilization process and three antipodals with unclear functions (Drews and Koltunow, 2011).

Although both of these cells harbor less condensed chromatin compared to that of the somatic cells, dimorphic epigenetic landscapes are established between the egg cell and the central cell, which is similar to that between the vegetative cell and the sperm cells, respectively, in the male gametophyte. The chromatin is largely decondensed in the central cell, which is characterized by low levels of DNA methylation due to DME demethylation activity, low levels of H3K9me2 and LHP1, while being transcriptionally active (Pillot et al., 2010; Baroux et al., 2011). By contrast, the egg cell chromatin is condensed, with high levels of H3K9me2 due to CMT3 activity and LHP1, coinciding with undetectable levels of active RNA PolII, reflecting a relatively transcriptional quiescent state (Pillot et al., 2010). Consequently, TE are highly active in the central cell while kept silent in the egg whereby a model of siRNA-based trans-signaling between the central cell and the egg cell has been proposed but it awaits demonstration (reviewed in Feng et al., 2013).

The dimorphic epigenetic state between the egg cell and the central cell is also reflected by the distinct core histone variants patterns established. Like that in the male gametes, both of the female gametes are devoid of most of the canonical, somatic H3 variants. The mature egg cell only harbors the H3.3 variant HTR5, while the central cell retains one H3.1 (HTR3) and two H3.3 variants (HTR8 and HTR14) (Ingouff et al., 2010). It was considered that the absence of H3.1 in the egg cell might be caused by the arrested cell cycle before S-phase, as H3.1 incorporation is linked with DNA synthesis (Ingouff et al., 2010; Stroud et al., 2012). The specific eviction of core histone H2B in the egg cell, rather than in the central cell, further underlies dimorphic chromatin composition between the gametes (Pillot et al., 2010).

3.4 Functions of chromatin dynamics during female gametogenesis

3.4.1 TE silencing to preserve genome integrity?

Likewise in sperm cells, a control over TE activity in the egg cell would be meaningful. It has been proposed that similar to the vegetative cell towards the sperm cells, the central cell may play a role in reinforcing TE silencing in the egg cell. Ibarra et al. (2012) uncovered that the central cell undergoes DNA demethylation mediated by DME from AT-rich, nucleosome-depleted euchromatic TEs, which in turn activates biosynthesis of corresponding siRNA. These siRNA may move to silence the homologous TEs via RdDM pathway in the egg cell, thereby enhance genome integrity of the next generation. However, how and when siRNA transposes to the egg cell remain elusive (Reviewed in Feng et al., 2013).

3.4.2 Epigenetic reprogramming in the egg towards totipotency in the zygote

The distinct chromatin states established in the egg cell and the central cell after cellularization of the female gametophyte reflect distinct epigenetic and transcriptional status. An interesting explanation for the transcriptionally quiescent state of the egg cell may be a role for establishing totipotency in the zygote that inherits the repressed transcriptional pattern from the egg cell likewise in animals (Seydoux and Braun, 2006). Unlike that of the egg cell, the chromatin state of the central cell will not be transmitted to the next generation. By contrast, the permissive chromatin state in the central cell will be passed to the nursing endosperm during fertilization, while the transcripts will be provide for the embryo development, thereby contributing to the acquisition of totipotence for the embryo.

3.4.3 Resetting the maternal epigenome

DNA demethylation in the central cell is, at least in part, mediated by DME (Ibarra et al., 2012). In addition, the Retinoblastoma Pathway is also passively involved in DNA demethylation in the central cell. The human Retinoblastoma protein (pRb), binded by the partner RbAp48, is known to inhibit gene expression via repressing S-phase gene

transcription (Nicolas et al., 2001). The interaction between the Arabidopsis homolog of pRb RETINOBLASTOMA RELATED 1 (RBR1) and of RbAp48 MULTICOPYSUPPRESSOR OF IRA1 (MSI1) in the MET1 promoter can repress MET1 transcription, thereby releasing the genes repressed by MET1 mediated DNA methylation (Jullien et al., 2008; Jullien and Berger, 2010). Thus, both passive and active mechanisms are employed to establish a permissive transcriptional state in the central cell, with activation of genes repressed by DNA methylation. The activation of maternally imprinted genes FWA and FIS2 (FERTILIZATION INDEPENDENT SEEDS2) in the central cell are depended on the derepression of MET1 through the Retinoblastoma pathway and DME activity, which will be inherited to the endosperm, thereby resulting in the maternal imprinting of these two genes (Choi et al., 2002; Kinoshita et al., 2004; Jullien et al., 2006). In addition, DME mediated DNA demethylation may be also required for activation of another maternal imprinted gene MEDEA (MEA) and the paternal imprinted gene PHERES1 (PHE1) (Xiao et al., 2003; Makarevich et al., 2008). The activated maternal MEA and FIS2 will facilitate the formation of FIS-PRC2 polycomb complex which recruits H3K27me3 to silence the maternal PHE1 allele, thereby contributing to the paternally imprinting of PHE1 (Kohler et al., 2003; Kohler et al., 2005). These evidences indicate that reprogramming of DNA methylation plays crucial roles in general genomic imprinting in the endosperm.

4. Conclusions and Future Prospects

Exciting findings in the past decades uncovered that epigenetic reprogramming conferred by dynamics of chromatin modifications, DNA methylation, nucleosome remodeling and small RNA regulation takes place throughout flowering plant sexual reproduction, which is likely to play multiple roles during plant sexual reproduction including the acquisition of distinct germline cell fate, resetting the epigenome of the germline to a ground state thereby promoting the acquisition of pluripotency, endowing the zygote with totipotency to develop into all cell types, as well as execution of cell division. It enlightened us on a better understanding of the mechanisms involved in sexual reproduction in addition to the genetic pathways. Although certain aspects on how epigenetic reprogramming operates sexual reproduction in flowering plants like small RNA regulation during male gametogenesis are

characterized, further efforts are needed for elucidating how epigenetic reprogramming regulates plant sexual reproduction. For instance, how the chromatin modifications are recruited or erased during sporogenesis, how the chromatin dynamics affects plant reproduction? Whether dynamics of DNA methylation is also involved during somatic to reproductive cell fate transition? Single-cell type based epigenome profiling coupled with next generation sequencing may pave the way for answering these questions, albeit this is hindered by the inaccessibility of the germline cells enclosed deep inside the reproductive organ. In addition, there are some other interesting aspects awaiting exploring, for instance, whether chromatin reprogramming also takes place during male sporogenesis? Do DNA methylation, histone modification and small RNA operate flowering plant reproduction via combinatory pathways? These points will pave the way for completing the landscape of epigenetic reprogramming involved in flowering plants.

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9. General discussion and outlook

9.1 General discussion

Plant sexual reproduction entails several fate transitions: from the somatic-to-reproductive cell fate during sporogenesis, from the haploid spores to the gametes during gametogenesis, and from the gametes to the embryo following fertilization (Grimanelli and Roudier, 2013). Accumulating evidences indicate that chromatin reprogramming that affects chromatin structure, conferred by dynamic changes in histone modifications, DNA methylation, nucleosome remodeling, and small RNA pathway, orchestrates these fate transitions, typically in post-meiotic gametophytic and embryo development (Baroux et al., 2007a; Baroux et al., 2007b; Ingouff et al., 2007; Schoft et al., 2009; Slotkin et al., 2009; Ingouff et al., 2010; Pillot et al., 2010; Autran et al., 2011; Baroux et al., 2011; Houben et al., 2011; Calarco et al., 2012; Ibarra et al., 2012; Jullien et al., 2012; Grimanelli and Roudier, 2013). However, whether chromatin reprogramming underlies the specification of SMCs, which is a key process before meiosis and marks the somatic-to-reproductive cell fate transition during plant sexual reproduction, remains largely unknown. The germline lineage in animals, primordial germ cells (PGCs) can be viewed as a functional equivalent of plant MMCs. While differentiation of PGCs is governed by extensive epigenetic reprogramming, which removes epigenetic barriers to achieve a ground-state of epigenome (Hajkovaa et al., 2002; Hajkova et al., 2008; Hackett et al., 2012). Importantly, genetic evidence indicates that small RNA dependent DNA methylation pathways regulate MMC specification in plants (Garcia-Aguilar et al., 2010; Olmedo-Monfil et al., 2010; Singh et al., 2011).

Thus, for my PhD, I aimed to explore whether chromatin reprogramming underlies the somatic-to-reproduction cell fate transition during the differentiation of MMC in *Arabidopsis* (Chapter II); which loci is targeted by chromatin reprogramming during MMC differentiation (Chapter III); whether chromatin reprogramming orchestrates PMC differentiation in *Arabidopsis* (Chapter IV); whether chromatin reprogramming is associated with MMC differentiation in the monocot plant rice (*Oryza sativa*) (Chapter V), as described in the Aims.

9.1.1 A robust method to quantitatively analyze chromatin modification and nuclear organization at single-cell level in whole-mount *Arabidopsis* ovules

Cytological and cytogenetic analyses of chromatin dynamics, at least for the female side, were rendered by the inaccessibility of MMC that embedded deeply inside the ovule. To overcome this technical limitation, we developed an efficient and robust method to quantitatively analyze MMC chromatin modification and nuclear organization at single-cell level in whole-mount *Arabidopsis* ovules (Chapter I). It is based on dissection and embedding of fixed ovules in miniature acrylamide pads directly on microscopic slides. The embedded ovules are then subjected to chemical and enzymatic treatments aiming at improving tissue clarity and permeability to the immunostaining reagents. The samples can be used for different downstream cytological analyses, including chromatin immunostaining, Fluorescence In-Situ Hybridization (FISH) and DNA staining for heterochromatin analysis. Confocal laser scanning microscopy (CLSM) imaging, with high resolution, followed by 3D reconstruction allows for quantitative measurements at single-cell resolution (She et al., 2014). This method enables qualitative and quantitative analysis of a large number of chromatin epitopes, with well preserved cellular and chromatin organization, DNA and protein epitopes, which was successfully used to describe chromatin organization in the female megaspore mother cell (She et al., 2013) and in the mature female gametophyte (Pillot et al., 2010; Autran et al., 2011). Compared to a recently published method for immunodetection from paraffin-embedded sections which takes more than 1.5 weeks, this protocol is short in time with only 3-4 days involved, and doesn't need the whole processes of sectioning for paraffin-embedded samples that may compromise the activity of antigens (Nic-Can et al., 2013).

9.1.2 Chromatin reprogramming underlies female somatic-to-reproductive cell fate transition in *Arabidopsis*

To resolve the question that whether chromatin reprogramming underlies the somatic-to-reproductive cell fate transition, we analyzed the nuclear organization and chromatin composition in differentiating MMCs of *Arabidopsis* using the method described in Chapter I,

and found that extensive chromatin reprogramming, conferred by chromatin decondensation, decrease of heterochromatin content, eviction of linker histones, changes of core histone variants and histone modifications, occurs during the somatic-to-reproductive cell fate transition in the ovule of *Arabidopsis*, with establishment of MMC specific chromatin that entails distinct epigenetic and transcriptional landscape. Similar chromatin dynamics was observed in ectopic MMCs of *ago9* mutant where gametophyte fate is ectopically expressed, suggesting that chromatin reprogramming marks the somatic-to-reproductive cell fate transition. By contrast to that in wild type *Arabidopsis*, H3K4me3 levels in MMCs are reduced in *sdg2* mutant, which impairs postmeiotic gametophyte development, albeit with normal meiotic progression, thus we infer that chromatin reprogramming is likely to contribute to establishing competence for postmeiotic gametophyte development.

While reprogramming of MMC chromatin is gradual and partially synchronous with meiotic S phase which lasts throughout MMC differentiation, with the replication of cytologically detectable heterochromatin precedes that of most of the euchromatin. It is of note that the followingly meiotic prophase progression and functional megaspore formation are conferred by additional chromatin reprogramming with both similar and specific dynamic changes of nuclear organization compared to that in differentiating MMCs, suggesting even broader coverage of chromatin reprogramming during plant sexual reproduction (Chapter II) (She et al., 2013).

9.1.3 Difficulty in developing a strategy enabling epigenome profiling of MMCs

Dynamic changes of chromatin modifications in differentiating MMC are marked by a reduction of the *Polycomb* silencing-associated mark H3K27me3 and increase of the *Trithorax* permissive transcription-associated mark H3K4me3 (She et al., 2013) (Chapter II). To identify the target loci affected by these epigenetic changes, we tried to perform comparative profiling of chromatin-immunoprecipitated fractions using antibodies against H2K27me3 and H3K4me3 modifications in the MMC and the nucellus (ChIP-seq). To isolate MMC nuclei for profiling, we tested two methods, including INTACT system and fluorescence assisted cell sorting (FACS). For the INTACT system, the target nuclei express a tag on their nuclear envelope (NTF). NTF is a fusion between the GFP fluorescent reporter, a

nuclear envelope protein and a biotin ligase recognition peptide (BLRP). Expression of biotin ligase (BirA) in the target cell will allow tagging the nuclei with biotin for further purification on streptavidin columns (Deal and Henikoff, 2010; 2011). We generated 4 *Arabidopsis* lines expressing the NTF specifically in the MMC and 3 lines for NTF in the nucellus, with MMC specific *KNUCKLES* promoter (Tucker et al., 2012), and the MMC/nucellus specific promoters selected from a published transcriptome profile data (Schmidt et al., 2011). However, none of these lines exhibit MMC or nucellus specific GFP signals on their nuclear envelope, which is possibly caused by the unstable activity of these promoters or due to integration of transgenes into transcriptional repressed chromatin region, while mRNA decay by post-transcriptional gene silencing (PTGS) may also contribute to the undetectable GFP signals in MMC/Nucellus-NTF lines (Chapter III). While, for FACS based on the pKNU-nlsYFP line, we tried to isolate MMC nuclei from pKNU-nlsYFP line by chopping the formaldehyde fixed flower buds into smashed pieces in the nuclei isolation buffer, and followed by filtration with 35 μm filter, the nuclei from the flower buds are with good quality and quantity, however, we failed to get the MMCs nuclei marked by YFP fluorescence, which was possibly due to YFP fluorescence quenching that induced by the reducing reagents in the buffer, while the difficulty in isolating MMCs nuclei from the attached surrounding tissue may also account for this (Chapter III).

9.1.4 Differentiating PMCs are marked by chromatin reprogramming in *Arabidopsis*

Similarly to MMC, pollen mother cells (PMCs) derive from somatic cells in the anther, which marks somatic-to-reproductive fate transition. This poses a fascinating question about whether PMCs specification is also governed by chromatin reprogramming. For this, we try to analyze nuclear organization and chromatin composition in differentiating PMCs of *Arabidopsis*, with the very preliminary results described in Chapter IV. We found that PMCs chromatin is distinct from the surrounding somatic cells, which is characterized by dynamic changes of chromatin organization, with loss of linker H1, eviction of histone variant H2A.Z, and distinct histone modification patterns compared to that in surrounding somatic cells, which is similar to that of MMC, suggesting extensive chromatin reprogramming involved in PMCs. To

confirm this, further analysis of chromatin composition and functional analysis of mutants where chromatin dynamics are affected would be necessary.

9.1.5 Evolutionarily conserved chromatin reprogramming among flowering plants?

Chromatin reprogramming has emerged as a fundamental regulator in SMCs differentiation in the dicot model plant *Arabidopsis* and in maize (Garcia-Aguilar et al., 2010; Olmedo-Monfil et al., 2010; Singh et al., 2011; She et al., 2013). However, little is described concerning that in other crop plants like rice (*Oryza sativa*), which feeds nearly 50% of the population over the world. To address this puzzle, we are trying to analyze nuclear organization and chromatin modifications in the monocot plant rice MMC, and characterized that the repressive marks H3K27me1 and H3K27me3 were both reduced in rice MMC, which is similar to that in *Arabidopsis* MMC, suggesting conserved chromatin dynamics may contribute to somatic-to-reproductive fate transition in different species (Chapter V). Future detailed comparative analyses of other histone marks and nuclear organization between rice MMC and surrounding nucellar cells will be required to unmask the epigenetic events entailed by rice MMC differentiation.

9.2 Outlook

Future efforts towards interpreting how chromatin reprogramming contributes to the somatic-to-reproductive cell fate transition in flowering plants

The fate transition from a somatic cell to reproductive cell entails global epigenomic changes, marked by drastic nuclear reorganization in *Arabidopsis* MMCs, which is central in post-meiotic development. Our preliminary results indicate that chromatin reprogramming is also associated with *Arabidopsis* PMCs specification and the monocot rice MMC differentiation, suggesting a broader role of chromatin reprogramming during the somatic-to-reproductive fate transition in different species. However, further analyses of nuclear organization and chromatin composition will be required to determine whether extensive chromatin reprograming is associated with PMCs and rice MMCs development. Analyses of

heterochromatin content will be important to illustrate the condensed/decondensed chromatin state in *Arabidopsis* PMCs and rice MMCs, while screening for other key chromatin marks including H3K4me2/3 and H3K9me1, and the histone variant like CENH3, as well as RNA PolII levels in differentiating PMCs and MMCs will be required for determining the epigenetic landscapes and transcriptional states of PMCs and rice MMCs. Functional analysis of mutants where chromatin dynamics is affected will be pivotal to uncover the roles of chromatin reprogramming during PMCs and rice MMC differentiation. These analyses will shed light on revealing the conserved or diverse chromatin reprogramming events between species.

Interpreting the mechanisms responsible for removal or loading of the histone modifications and histone variants in differentiating SMCs will pave the way for understanding how chromatin reprogramming operates somatic-to-reproductive cell fate transition. Chromatin reprogramming that confers changes of chromatin structure, is likely to influence the transcriptional pattern, hence the cell function. Thus, characterization of the interplay between chromatin dynamics and transcriptional networks in differentiating SMCs will be pivotal for understanding the functional significance of reprogramming events that orchestrates reproductive cell fate acquisition, this can be achieved by epigenome profiling of SMCs at single-cell resolution. However, at least for the female side, this was impeded by the highly diluted MMCs enclosed within complex floral organs. Dynamic patterns of DNA methylation have been described to operate post-meiotic gametophyte development and embryo development in plants, but its role during SMCs specification remains elusive. Single-cell based sequencing of methylated sites in CG, CHG, and CHH contexts, will provide the detailed information concerning the process of SMCs differentiation that governed by DNA methylation at high resolution.

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11. Appendix

Table 1. Primers used for this study.

Vector	**Entry-NTF (SHE1)**
amplicon	NTF coding sequence
primers	ws1: AAAAAGCAGGCTATGAATCATTCAGCGAAAACC
	ws2: AGAAAGCTGGGTTCAAGATCCACCAGTATCCTCA
	ws3: GGGGACAAGTTTGTACAAAAAAGCAGGCT (attB1 adapter)
	ws4: GGGGACCACTTTGTACAAGAAAGCTGGGT (attB2 adapter)
cloning sites	attB1, attB2
Vector	**pAT5G14980-NTF (SHE3)**
amplicon	AT5G14980 promoter
primers	ws5: GCTCTAGAGCTATCAGAGAATCATAAAAAGGAGAC
	ws6: GCTCTAGAGCTTTAGTAGTACCGTTAATTAAGATG
cloning sites	xbaI
Vector	**pAT3G52160-NTF (SHE4)**
amplicon	AT3G52160 promoter
primers	ws7: GCTCTAGAGCTTCATTGTAGAGATCCGTTGG
	ws8: GCTCTAGAGCCACTAAGCATCAAAACCTTGTG
cloning sites	xbaI
Vector	**pAT2G03740-NTF (SHE5)**
amplicon	AT2G03740 promoter
primers	ws9: GCTCTAGAGCTCCCTTATAGTTGGACCATCG
	ws10: GCTCTAGAGCATGTGTGTTTTTGGGAGTAAGC
cloning sites	xbaI
Vector	**pAT2G24500-NTF (SHE6)**
amplicon	AT2G24500 promoter
primers	ws17: GCTCTAGAGCTCCCATCTTTCTCTATCTCAG
	ws18: GCTCTAGAGCTTGTCTTTCTCGTTGTTGCT
cloning sites	xbaI
Vector	**pAT1G72320-NTF (SHE8)**
amplicon	AT1G72320 promoter
primers	ws13: GCTCTAGAGCCAGCTTTATACTAGGAACGTGC
	ws14: GCTCTAGAGCCTTTGAGGATGCAAAAACAC
cloning sites	xbaI

Vector	**pAT1G11270-NTF (SHE9)**
amplicon	AT1G11270 promoter
primers	ws15: GCTCTAGAGCGCAGACTGAAATGTAATACCAGC
	ws16: GCTCTAGAGCCACGAGTTGAGCTTAAGGAAAT
cloning sites	xbaI
Vector	**pAT5G14010-NTF (p*KNUCKLES*-NTF, SHE10)**
amplicon	*KNUCKLES* promoter
primers	ws28: GCTCTAGAGCTGGTAGATTTGTTCTGTGCATCCTA
	ws29: AGGCGCGCCTTTTTGAGAGGTTCTTAAGCTACAGAGGA
cloning sites	xbaI, AscI
Vector	**pAT5G14010-EGFP (p*KNUCKLES*-EGFP, SHE11)**
amplicon	*KNUCKLES* promoter
primers	ws30: CCCAAGCTTGGGTGGTAGATTTGTTCTGTGCATCCTA
	ws31: CGGATCCCATTTTTGAGAGGTTCTTAAGCTACAGAGGA
cloning sites	HindIII, BamH1

Table 2. Vectors used for this study.

Vector	Backbone	Insert	Original Name	Host Resistance
SHE1	pDONOR221	NTF coding region	Entry-NTF	Kanamycin
SHE2	pMOA36	NTF coding region	pQAN-NTF	Spectinomycin
SHE3	SHE2	promoter of AT5G14980	pAT5G14980-NTF	Spectinomycin
SHE4	SHE2	promoter of AT3G52160	pAT3G52160-NTF	Spectinomycin
SHE5	SHE2	promoter of AT2G03740	pAT2G03740-NTF	Spectinomycin
SHE6	SHE2	promoter of AT2G24500	pAT2G24500-NTF	Spectinomycin
SHE8	SHE2	promoter of AT1G72320	pAT1G72320-NTF	Spectinomycin
SHE9	SHE2	promoter of AT1G11270	pAT1G11270-NTF	Spectinomycin
SHE10	SHE2	promoter of *KNUCKLES*	p*KNUCKLES*-NTF	Spectinomycin
SHE11	pCB72	promoter of *KNUCKLES*	pKNU-EGFP	Kanamycin

Table 3. List of seed stock used for this study.

Name	Genotype	Mother line	Father line	Population Info	Remarks
SHE1	RPS5A:hBirA/RPS5A:hBirA	RPS5A:hBirA		homozygous selfed	From A. Boyko
SHE2	GL2:BirA/GL2:BirA, ACT2:NTF/ACT2:NTF	GL2:BirA/GL2:BirA	ACT2:NTF/ ACT2:NTF	homozygous	From C. Baroux (from Deal and Henikoff)
SHE3	pAT5G14980-NTF	col		T1 transformants	
SHE4	pAT3G52160-NTF	col		T1 transformants	

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SHE5	AT2G03740-NTF	col		T1 transformants	
SHE6	pAT2G24500-NTF	col		T1 transformants	
SHE8	pAT1G72320-NTF	col		T1 transformants	
SHE9	pAT1G11270-NTF	col		T1 transformants	
SHE10	p*KNUCKLES*-NTF	col		T1 transformants	
SHE11	pKNU-EGFP	col		T1 transformants	
SHE12	atxr5/atxr5, atxr6/atxr6	atxr5 (SALK_130607)	atxr6 (SAIL_240_H01)	homozygous	From Scott D. Michaels
SHE13	suvh2/suvh2	suvh2		homozygous	From C. Baroux
SHE14	fas2-4/+	fas2-4/+		heterozygous	From C. Baroux (salk line)
SHE15	pLHP1:LHP1-EGFP/ pLHP1:LHP1-EGFP	pLHP1:LHP1-EGFP		homozygous	From C. Baroux
SHE16	ago9-4/9-4	ago9-4		homozygous	From C. Baroux (from JP Calzada)
SHE17	pHTR12:HTR12-EGFP/pHTR12:HTR12-EGFP	pHTR12:HTR12-EGFP		homozygous	From C. Baroux (from Spector's lab)
SHE18	HTA11:GFP/HTA11:GFP	HTA11:GFP		homozygous	From C. Baroux
SHE19	sdg2-1/+(24.43p)			heterozygous	From W. Shen
SHE20	sdg2-1/+(19k)			heterozygous	From W. Shen
SHE21	H1.1-GFP/H1.1-GFP	H1.1-GFP		homozygous	From C. Baroux
SHE22	LHP1-GFP/LHP1-GFP	LHP1-GFP		homozygous	From C. Baroux
SHE23	KNU-nlsYFP	KNU-nlsYFP		homozygous	From C. Baroux (from Koltunow group)
SHE24	ago9-4/+; pH1.2::H1.2-GFP	ago9-4	pH1.2::H1.2-GFP	heterozygous	From C. Baroux
SHE25	ago9-4/+; pH1.1::H1.1-EGFP	ago9-4	pH1.1::H1.1-EGFP	heterozygous	From C. Baroux
SHE29	sdg2				T-DNA mutant from ABRC
SHE30	ref6-1/ref6-1			homozygous	From X. Cao (salk line Salk-001018)
SHE31	ref6-3/ref6-3			homozygous	From X. Cao (salk line SAIL747A07)
SHE32	atx1/atx1	atx1		selfed homozygous	From S. Pien
SHE33	kyp2/kyp2			homozygous	From C. Baroux (CS6367)
SHE50	sdg2/sdg2,pH1.1:H1.1-GFP/pH1.1:H1.1-GFP	pH1.1:H1.1-GFP	sdg2/sdg2	homozygous for sdg2	
SHE51	sdg2/sdg2, pKNU-nlsYFP	pKNU-nlsYFP	sdg2/sdg2	homozygous for sdg2	